FUNDAMENTAL UNDERSTANDING OF THE BIOCHEMICAL CONVERSION OF *BUDDLEJA DAVIDII* TO FERMENTABLE SUGRAS

A Dissertation
Presented to
The Academic Faculty

by

Bassem Bishara Hallac

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy in the
School of Chemistry and Biochemistry

Georgia Institute of Technology

May, 2011

FUNDAMENTAL UNDERSTANDING OF THE BIOCHEMICAL CONVERSION OF *BUDDLEJA DAVIDII* TO FERMENTABLE SUGRAS

Approved by:

Dr. Arthur J. Ragauskas, Advisor
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Preet Singh
School of Materials Science and Engineering
Georgia Institute of Technology

Dr. Charles L. Liotta
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Yulin Deng
School of Chemical and Biomolecular Engineering
Georgia Institute of Technology

Dr. Julia Kubanek
School of Chemistry and Biochemistry
Georgia Institute of Technology

Date Approved: March 22, 2011

قال الإمام الشافعي:

بقَدْرِ الكَدِّ تُكتَسَــبُ المَعَــالي ومَنْ طَلَبَ العُلا سَهِرَ اللّيالي
ومَنْ رامَ العُلا مِنْ غَيرِ كَــدٍّ أضَاعَ العُمرَ في طَلَبِ المُحَالِ
تَرُومُ العِــزَّ ثــم تَنَــامُ لَــيلاً يَغُوصُ البَحْرَ مَن طَلَبَ اللآلي

DEDICATION

This dissertation is dedicated to my family, who has always been there for me, as I hurdled all the obstacles during the completion of this research work. My family has given me nothing but love, care, encouragement, support, and advice throughout the course of my doctoral research.

Thank you,

Mom and Dad

Brothers Boutros and Basseem

Grandmother Emerance

Aunt Mary

Uncle Robert

Aunt Rima

Cousins Ramzi, Rami, and Ranya

ACKNOWLEDGEMENTS

During the initial phases of graduate school, earning my Ph.D. degree seemed a dream far away and a goal that I would only hope to achieve. With the love, care, support, and sincere advice from many individuals, this dissertation was made possible. I would like to say 'thank you' to all of them.

First and foremost, I would like to acknowledge my advisor, Dr. Arthur J. Ragauskas, for his guidance, encouragement, advice, and mentorship throughout my doctoral research. The knowledge I gained from him will greatly help me in my future endeavors. I would also like to thank my thesis committee, Dr. Charles L. Liotta, Dr. Julia Kubanek, Dr. Preet Singh, and Dr. Yulin Deng, for their insightful comments and support from the initial to the final level of this project. My sincere thanks go out to Dr. Richard J. Murphy of Imperial College London, who helped me immensely with my studies and invited me to work in his lab for one month.

I am grateful to my co-workers at Georgia Tech, especially Dr. Poulomi Sannigrahi, Dr. Yunqiao Pu, Shaobo Pan, Matyas Kosa, and Mikhail Levit; and Dr. Michael J Ray, at Imperial College, for their support in many aspects during the completion of this project.

I owe my deepest gratitude to Dr. Christopher White for helping me pursue my higher education. Without his initial support, I would not have had the opportunity to come to Georgia Tech for my graduate studies.

I also would like to acknowledge the financial support from the Paper Science and Engineering Fellowship program at the Institute of Paper Science and Technology.

Finally, I would like to thank all of my friends for the good times and lasting memories we shared together.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS V

LIST OF TABLES XI

LIST OF FIGURES XV

LIST OF EQUATIONS XIX

LIST OF ABBREVATIONS XX

SUMMARY XXIII

CHAPTER 1: INTRODUCTION 1

CHAPTER 2: LITERATURE REVIEW 4
2.1 Problem Statement 4
2.2 Cellulosic Ethanol 7
2.2.1 Bioresources 8
2.2.1.1 *Buddleja davidii* 9
2.2.2 Lignocelluloses 14
2.2.2.1 Lignin 16
2.2.2.2 Hemicelluloses 24
2.2.2.3 Cellulose 27
2.2.2.3.1 Isolation of Cellulose for DP Measurements 29
2.2.2.3.2 Determining Cellulose Degree of Polymerization 31
2.2.2.3.3 Level-Off Degree of Polymerization 37
2.2.2.3.4 Light Scattering Measurements of Cellulose DP 39
2.2.3 Pretreatment 42
2.2.3.1 Pretreatment Methods 44
2.2.3.1.1 Alkali Pretreatment 44
2.2.3.1.2 Dilute Acid Pretreatment 52
2.2.3.1.3 Organosolv Pretreatment 56
2.2.3.2 Inhibition 59
2.2.3.3 Effect of Pretreatment on the Cellulose Degree of Polymerization 63
2.2.3.3 Effect of Pretreatment on Cellulose Crystallinity 67
2.2.3.4 Effect of Pretreatment on Surface Area and Pore Volume of Biomass 71

CHAPTER 3: EXPERIMENTAL MATERIALS AND PROCEDURES 75
3.1 Materials 75
3.1.1 Chemicals 75
3.1.2 Biomass Preparation 75
3.1.2.1 *Buddleja davidii* Samples for Characterization Experiment 75

3.1.2.2 *Buddleja davidii* for Ethanol Organosolv Pretreatment Experiment 75
3.2 Experimental Procedures 76
3.2.1 Dichloromethane (DCM) Extraction 76
3.2.2 Holocellulose Pulping 77
3.2.3 Isolation of Cellulose, Hemicellulose, and Milled Wood Lignin 77
3.2.4 Ethanol Organosolv Pretreatment 78
3.3 Analytical Procedures 80
3.3.1 Carbohydrates and Lignin Content 80
3.3.2 Ash Analysis 80
3.3.3 Enzymatic Hydrolysis 80
3.3.4 NMR Spectroscopy 81
3.3.4.1 Solid-State CP/MAS ^{13}C NMR Characterization of Cellulose 81
3.3.4.2 Quantitative ^{13}C NMR Characterization of Lignin 82
3.3.4.3 Quantitative ^{31}P NMR Characterization of Lignin 82
3.3.4.4 DEPT-135 ^{13}C NMR Characterization of Lignin 83
3.3.4.5 Qualitative ^{13}C-^{1}H 2D HSQC Characterization of Lignin 83
3.3.5 Gel Permeation Chromatography Analysis of Cellulose 83
3.3.6 Gel Permeation Chromatography Analysis of Lignin 84
3.3.7 Gel Permeation Chromatography Analysis of EOL 85
3.3.8 Error Analysis 86

CHAPTER 4: BIOMASS CHARACTERIZATION OF *BUDDLEJA DAVIDII*: A POTENTIAL FEEDSTOCK FOR BIOFUEL PRODUCTION 87
4.1 Introduction 87
4.2 Experimental Section 89
4.2.1 Sample Preparation 89
4.2.2 Ash and Extractive Contents 90
4.2.3 Tannin Content 90
4.2.4 Heating value 90
4.2.5 GC/MS Analysis 90
4.2.6 Carbohydrate and Lignin Composition 91
4.2.7 Holocellulose Pulping 91
4.2.8 Cellulose Analysis 91
4.2.9 Hemicellulose Isolation 91
4.2.10 Lignin Structural Analysis 92
4.2.11 Gel Permeation Chromatography Analysis of Cellulose 92
4.2.12 Gel Permeation Chromatography Analysis of Lignin 92
4.3 Results and Discussion 92
4.3.1 Ash and Extractive Analyses 92
4.3.2 Biomass Composition 97
4.3.3 Cellulose Analysis 98
4.3.4 Structural Analysis of *B. davidii* MWL Using Quantitative ^{13}C NMR 100
4.3.5 Structural Analysis of *B. davidii* MWL Using Quantitative ^{31}P NMR 103
4.3.6 GPC Analysis of *B. davidii* MWL 105
4.4 Conclusions 106

CHAPTER 5: EFFECT OF ETHANOL ORGANOSOLV PRETREATMENT ON ENZYMATIC HYDROLYSIS OF *BUDDLEJA DAVIDII* STEM BIOMASS 107
5.1 Introduction 107
5.2 Experimental Section 111
5.2.1 Biomass Material Preparation 111
5.2.2 Ethanol Organosolv Pretreatment 111
5.2.3 Analytical Procedures 111
5.2.4 Enzymatic Hydrolysis 112
5.2.5 Cellulose Characterization 112
5.3 Results and Discussion 113
5.3.1 Ethanol Organosolv Pretreatment of *Buddleja davidii* 113
5.3.2 Enzymatic Hydrolysis of Organosolv Pretreated *Buddleja davidii* 115
5.3.3 Crystallinity and Ultrastructure of *Buddleja davidii* Cellulose 118
5.4 Conclusions 123

CHAPTER 6: CHEMICAL TRANSFORMATIONS OF *BUDDLEJA DAVIDII* LIGNIN DURING ETHANOL ORGANOSOLV PRETREATMENT 124
6.1 Introduction 124
6.2 Experimental Section 127
6.2.1 Biomass Material Preparation 127
6.2.2 Ethanol Organosolv Pretreatment 127
6.2.3 Structural Analysis of Ethanol Organosolv Lignin 128
6.2.4 Gel Permeation Chromatography Analysis of EOL 128
6.3 Results and Discussion 128
6.3.1 Delignification Response 128
6.3.2 Elemental and Chemical Composition of EOL 129
6.3.3 Gel-Permeation Chromatography (GPC) Analysis of *B. davidii* EOL 130
6.3.3 Structural Analysis of *B. davidii* EOL Using Quantitative ^{13}C, DEPT-135 and 2D NMR 132
6.3.4 Structural Analysis of *B. davidii* EOL Using Quantitative ^{31}P NMR 143
6.4. Conclusions 145

CHAPTER 7: CORRELATION BETWEEN ANATOMICAL CHARACTERISTICS OF ETHANOL ORGANOSOLV PRETREATED *BUDDLEJA DAVIDII* AND ITS ENZYMATIC CONVERSION TO GLUCOSE 147
7.1 Introduction 147
7.2 Experimental Section 149
7.2.1 Preparation of Biomass 149
7.2.2 Enzymatic Hydrolysis 149
7.2.3 Embedding 150
7.2.4 Sectioning and Staining 150
7.2.5 Light Microscopy Imaging and Analysis 150
7.3 Results and Discussion 152
7.4. Conclusions 159

CHAPTER 8: OVERALL CONCLUSIONS 160

CHAPTER 9: RECOMMENDATIONS FOR FUTURE WORK 164

APPENDIX A: GLOSSARY 165

APPENDIX B: COVER IMAGE 166

APPENDIX C: COPYRIGHT PERMISSIONS 167

REFERENCES 207

LIST OF TABLES

Table 1. Average productivity of several biomass species in the United States..... 9

Table 2. Cell wall macromolecule composition of different softwood and hardwood species..... 15

Table 3. Proportions of different types of lignin interlinkages per 100 Aromatic units in softwood and hardwood lignin..... 21

Table 4. Molar percentage of guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units in several biomass lignin..... 21

Table 5. Sugar and lignin analysis of spruce LCC fractions..... 23

Table 6. The major hemicelluloses components in softwood and hardwood..... 27

Table 7. Hemicellulose composition and DP in several biomass..... 27

Table 8. Cellulose crystallinity and ultrastructure from several biomass determined by CP/MAS ^{13}C NMR spectroscopy..... 29

Table 9. DP of native wood and non-woody celluloses after nitration using the viscometric method..... 34

Table 10. DP of native wood and non-woody celluloses..... 37

Table 11. Typical level-off degree of polymerization of some cellulose materials..... 39

Table 12. DP of several cellulose-rich samples after dissolution in LiCl/DMI using the SEC-MALLS method. 41

Table 13. Comparison between four alkali reagents based on cost, handling, efficiency in enhancing biomass digestibility, and recovery. 48

Table 14. Recommended lime pretreatment conditions for baggase, wheat straw, switchgrass, and corn stover..... 49

Table 15. Optimum dilute sulfuric acid pretreatment conditions for several biomass, sugar yields, and the efficiency of enzymatic hydrolysis. 54

Table 16. Glucan, xylans, and klason lignin content in untreated and dilute H_2SO_4 pretreated corn stover, switchgrass, and poplar 55

Table 17. Compositional analysis of the raw materials and the three fractions after ethanol organosolv pretreatment of poplar and pine at the optimum conditions..... 58

Table 18. Weight-average molecular weight ($\overline{M}_n$), the number-average molecular weight ($\overline{M}_n$), polydispersity index (D) ($\overline{M}_w / \overline{M}_n$), and functional groups of pine and poplar EOL..... 59

Table 19. DP of several biomass cellulose after various pretreatment technologies..... 64

Table 20. Change in DP of wheat straw cellulose after various organosolv pretreatment 66

Table 21. Crystallinity index of several untreated and pretreated biomass cellulose using various pretreatment technologies. 68

Table 22. Crystallinity of ethanol organosolv pretreated substrates prepared from lodgepole pine under varied conditions 69

Table 23. Effect of acid pretreatment on the specific surface area of several biomass 72

Table 24. Effect of caustic, peracetic acid, and ethylene glycol pretreatment on the specific surface area of wheat straw. 73

Table 25. Accessible pore volume of untreated and dilute acid pretreated corn stover ... 74

Table 26. Process conditions for the ethanol organosolv pretreatment of *Buddleja davidii* 79

Table 27. Ash and extractive contents in the leaves, bark, and stem of native *Buddleja davidii*. 93

Table 28. Inorganic trace elements profile for the leaves, bark, and stem of native *Buddleja davidii*. 93

Table 29. Summary of the heating values of the leaves, bark, and stem of native *Buddleja davidii*. 94

Table 30. Heating values, and lignin and extractive contents of *Buddleja davidii* and different wood species. 94

Table 31. Evaluation of dichloromethane (DCM) extracts by GC-MS from the leaves, bark, and stem of native *Buddleja davidii*. 96

Table 32. Cell wall macromolecule composition of *Buddleja davidii* and other wood species, including lignin, cellulose and hemicelluloses. 97

Table 33. Carbohydrate profile and lignin content in native *Buddleja davidii* stem, bark, and leaves. 98

Table 34. Assignments of signals in the C-4 region of CP/MAS ^{13}C NMR spectrum obtained for native *Buddleja davidii* cellulose 100

Table 35. Quantification of several lignin moieties in *Buddleja davidii* milled wood lignin using ^{13}C NMR. 102

Table 36. Quantification of several hydroxyl functional groups in *Buddleja davidii* milled wood lignin using ^{31}P NMR 105

Table 37. Comparison between hydroxyl content in *Buddleja davidii* milled wood lignin and other wood species. 105

Table 38. Comparison between the weight-average molecular weight ($\overline{M}_w$), the number-average molecular weight ($\overline{M}_n$), and the polydispersity index (D) ($\overline{M}_w / \overline{M}_n$) of *Buddleja davidii* milled wood lignin and other species. 106

Table 39. Compositional analysis of the three fractions after the ethanol organosolv pretreatment of *Buddleja davidii* at various conditions. 113

Table 40. Gel permeation chromatography (GPC) analysis of untreated and three organosolv pretreated *Buddleja davidii* cellulose samples 117

Table 41. Results of spectral fitting of the C-4 region of CP/MAS ^{13}C NMR spectrum of cellulose A. 119

Table 42. Comparison between the crystallinity and ultrastructure of cellulose isolated from untreated and ethanol organosolv pretreated *Buddleja davidii*. 120

Table 43. Elemental analysis and chemical composition of MWL and EOL of *Buddleja davidii* 130

Table 44. Weight-average molecular weight ($\overline{M}_w$), number-average molecular weight ($\overline{M}_n$), and polydispersity index ($\overline{M}_w / \overline{M}_n$) of MWL and EOL. 131

Table 45. Quantitative comparison between MWL and EOL based on the ^{13}C NMR spectra. 133

Table 46. Assignment of the expanded 160–50 ppm region of the quantitative ^{13}C NMR spectra. 139

Table 47. Hydroxyl content of MWL and EOL of *Buddleja davidii* as determined by quantitative ^{31}P NMR..... 144

LIST OF FIGURES

Figure 1. Global petroleum consumption from 1987 until 2010. 4

Figure 2. Global CO_2 emissions from the consumption of petroleum from 1980 to 2008..... 6

Figure 3. Data summary of the world crude oil prices from 1987 to 2010..... 6

Figure 4. An overview of the bioconversion of lignocellulosic biomass to ethanol: 1st and 2nd generation bioethanol. 8

Figure 5. Distribution of *Buddleja davidii* in the U.S..... 10

Figure 6. Images of *Buddleja davidii* that illustrate its growth habitat. a, vigorous growth in open and poor soil. b, growth in very poor sites. c, growth in no soil at all. 11

Figure 7. Examples of some compounds isolated from *Buddleja* that have anti-inflammatory and antifungal activity. 1 and 2 are a trisaccharide and disaccharide, respectively with phenylethanoid and caffeic acid attachments; 3, 4, and 5 are flavonoids; and 6 is buddledin A. 13

Figure 8. Schematic representation of the secondary cell wall containing cellulose, hemicelluloses and lignin..... 14

Figure 9. Schematic representation of plant cell wall..... 16

Figure 10. Example for structure of native softwood lignin. 17

Figure 11. Model for poplar milled wood lignin. 18

Figure 12. Some common linkages found in lignin formed via radical coupling between phenylpropane units. 20

Figure 13. Structure of ester linkage between lignin and glucuronoxylan. 22

Figure 14. Structure of ether linkage between lignin and glucomannan. 23

Figure 15. Principal structure of: (I) arabinoglucuronoxylan in softwood, (II) galactoglucomannans in softwood, and (III) glucuronoxylan in harwood. 25

Figure 16. Molecular structure of cellulose. 28

Figure 17. Cellulose morphology. 42

Figure 18. Schematic of goals of pretreatment on lignocellulosic material. 43

Figure 19. Sugar yields from lime pretreated bagasse, switchgrass, and corn stover after enzymatic hydrolysis. 49

Figure 20. Composition of untreated and lime pretreated baggasse, switchgrass, and corn stover. 50

Figure 21. Composition of untreated and lime pretreated poplar; and the sugar yields after enzymatic hydrolysis. 51

Figure 23. Effect of xylan removal on the enzymatic digestibility of corn stover after dilute acid pretreatment. 53

Figure 22. Acid-catalyzed hydrolysis of xylan. 53

Figure 24. Reactions occurring during hydrolysis of lignocellulosic materials [136]..... 60

Figure 25. Schematic representation for the formation of HMF, formic and levulinic acids from cellulose during pretreatment. 61

Figure 26. The effect of the ethanol organosolv pretreatment severity on the production of furans from *Miscanthus x giganteus*. 62

Figure 27. The relationship between crystallinity of poplar and its enzymatic digestibility. 71

Figure 28. Soxhlet extractor used for dichloromethane (DCM) extraction of *Buddleja davidii* stems, bark, and leaves. 76

Figure 29. The process of a typical ethanol production scheme from biomass. 89

Figure 30. Solid-state CP/MAS ^{13}C NMR spectrum of native *Buddleja davidii* cellulose. 98

Figure 31. Spectral fitting for the C-4 region of CP/MAS ^{13}C NMR spectrum of native *Buddleja davidii* cellulose. 99

Figure 32. Quantitative ^{13}C NMR spectrum of milled wood lignin from native *Buddleja davidii*. 102

Figure 33. DEPT-edited ^{13}C NMR spectrum of milled wood lignin from native *Buddleja davidii*. 103

Figure 34. Quantitative ^{31}P NMR of milled wood lignin from native *Buddleja davidii*. 104

Figure 35. Enzymatic hydrolysis of untreated and ethanol organosolv pretreated *Buddleja davidii*. 114

Figure 36. Solid-state CP/MAS ^{13}C NMR spectrum of *Buddleja davidii* cellulose isolated from the substrate prepared at condition A (180°C, 60 min, 1.25% H_2SO_4, and 50% ethanol). 118

Figure 37. Spectral fitting for the C-4 region of the CP/MAS ^{13}C NMR spectrum of cellulose isolated from substrate A. 119

Figure 38. Extent of ethanol organosolv delignification at various pretreatment conditions. 129

Figure 39. Quantitative ^{13}C NMR spectra of *B. davidii* MWL and EOL 3. 132

Figure 40. Expanded 160–50 ppm region of quantitative ^{13}C NMR spectra *B. davidii* MWL and EOL 3. 136

Figure 41. DEPT–135 ^{13}C NMR spectra of *B. davidii* MWL and EOL 3. 137

Figure 42. HSQC 2D NMR spectra of *B. davidii* MWL (A and B) and EOL 3 (C and D). 138

Figure 43. Lignin substructures identified in *B. davidii* MWL and EOL. 140

Figure 44. Quantitative ^{31}P NMR spectra of *B. davidii* MWL and EOL 3. 144

Figure 45. Rating system used to categorize the cell structural features of untreated and pretreated *B. davidii*. 151

Figure 46. Quantitative representation of the characteristics of the untreated and pretreated *B. davidii*. 152

Figure 47. Enzymatic conversion of *Buddleja davidii* cellulose to glucose after ethanol organosolv pretreatment carried under different conditions. 153

Figure 48. Effect of ethanol organosolv pretreatment on the cell structure of *Buddleja davidii*. 155

Figure 49. Polarized light images of ethanol organosolv pretreated *Buddleja davidii* under condition 3. 158

LIST OF EQUATIONS

Equation 1. Formation of *β*-O-4 interlinkage via radical coupling. 21

Equation 2. Reaction mechanism of chlorine dioxide with phenolic hydroxyl lignin..... 30

Equation 3. Acid-catalyzed hydrolysis of cellulose. 38

Equation 4. Mechanism for the peeling reaction of cellulose under alkali condition..... 45

Equation 5. Mechanism for the base-catalyzed cleavage of cellulose. The $S_N$icB(2) refers to a nucleophilic substitution by an internal nucleohpile – the conjugate base of the C2 hydroxyl group. 46

Equation 6. Base-catalyzed removal of acetate groups from xylan. 47

Equation 7. Derivatization of phenolic structures with 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP). 104

Equation 8. An example of lignin condensation reaction during acid-catalyzed treatment of biomass. 130

Equation 9. Degradation mechanism of *β*-aryl ether (*β*-*O*-4') during ethanol organosolv pretreatment. 141

LIST OF ABBREVATIONS

4-O-MeGlcA	4-O-methylglucuronic acid
Ara	Arabinose
B. davidii	*Buddleja davidii*
$CDCl_3$	Deuterated Chloroform
CBH	Cellobiohydrolase
CP/MAS	Cross polarization/magic angle spinning
CrI	Crystallinity index
D	Polydispersity index
DC	Degree of condensation
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DMSO-d_6	Deuterated dimethyl sulfoxide
DP	Degree of polymerization
DP_v	Viscosity-average degree of polymerization
DP_w	Weight-average degree of polymerization
DPX	di-n-butyl phthalate in xylene
ELSD	Evaporative light scattering detector
EOL	Ethanol organosolv lignin
EOP	Ethanol organosolv pretreatment
FAA	Formalin-acetic acid-alcohol
FPU	Filter paper unit

F_{RE}	Fraction of reducing ends
FWHH	Full width at half-height
g	guaiacyl
Gal	Galactose
GalA	Galacturonic acid
GC	Gas Chromatography
Glu	Glucose
GPC	Gel permeation chromatography
h	*p*-hydroxyphenyl
HCl	Hydrochloric acid
HMF	5-hydroxylmethyl furfural
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HPLC	High performance liquid chromatography
H_2SO_4	Sulfuric acid
HSQC	Heteronuclear single quantum coherence
HW	Hardwood
ICP	Inductively coupled plasma
IU	International unit
LAP	Laboratory analytical procedure
LCC	Lignin-carbohydrate complex
Man	Mannose
ML	Middle lamella
$\overline{M}_n$	Number-average molecular weight

$\overline{M}_w$	Weight-average molecular weight
ML	Middle lamella
MS	Mass spectrometry
MSTFA	N-methyl-n-trimethylsilytrifluoroacetamide
MWD	Molecular weight distribution
MWL	Milled wood lignin
NREL	National renewable energy laboratory
NMR	Nuclear magnetic resonance
od	Oven dry
P_2O_5	Phosphorous pentoxide
ppm	Parts per million
PTFE	Polytetrafluoroethylene
RSD	Relative standard deviation
s	syringyl
SSA	Specific surface area
SW	Softwood
TAPPI	Technical association of the pulp and paper industry
THF	Tetrahydrofuran
TMDP	2-chloro-4,4,5,5-tetramethyl-1,3,2dioxaphospholane
T. reesei	*Trichoderma reesei*
UV	Ultraviolet
Xyl	Xylose
ZrO_2	Zirconium Oxide

SUMMARY

Bioethanol is becoming one of the leading substitutes for fossil fuel and is being commercially produced from corn and sugarcane. Lignocellulosic bioethanol is currently being explored as an alternative to corn/sugarcane ethanol because the former is not involved with the "fuel or food" issue. Many biomass sources are being examined but the importance is to find those with attractive agro-energy features. Thus, there is always a necessity to broaden the bioresource base. In addition, cellulosic ethanol production is more challenging than corn/sugarcane ethanol production because biomass is resistant to chemical and biological degradation. To reduce the recalcitrant nature of biomass, a pretreatment stage is required but it is the most intensive operating/operating cost component of cellulosic ethanol production. Therefore, research is heavily focused on understanding the effect of pretreatment technologies on the fundamental characteristics of lignocellulosic biomass.

The first study in the thesis investigates *Buddleja davidii* as a potential biomass source for bioethanol production. The work focuses on the determination of ash, extractives, lignin, hemicellulose, and cellulose content in this plant, as well as detailed elucidation of the chemical structures of both lignin and cellulose by NMR spectroscopy. *B. davidii* has several unique agro-energy features ranging from its distribution and wide range of growth habitat to aspects of its composition (cellulose DP of 100), as well as some undesired characteristics such as, relatively high lignin (30%) and hemicellulose (34%) contents, low cellulose content (35%), and a high cellulose crystallinity index (0.55).

To determine the balance between these positive and negative factors on potential glucose yields, evaluation and enzymatic hydrolysis behavior of *B. davidii* was needed. The second study presents research on the ethanol organosolv pretreatment of *B. davidii* and its ability to produce enzymatically hydrolysable substrates. Furthermore, the study explored the fundamental characteristics of pretreated *B. davidii* in the context of developing an efficient bioconversion of cellulose to glucose. The presence of high lignin and hemicellulose contents in *B. davidii* wood was not found to be a negative factor since these biopolymers were easily removed during ethanol organosolv pretreatment (EOP). It was also concluded that the removal of hemicellulose, delignification, reduction in DP of cellulose, and the conversion of crystalline cellulose dimorphs (I_α/I_β) to the easily degradable *para*-crystalline and amorphous celluloses were the characteristics accounted for efficient enzymatic deconstruction of *B. davidii* after EOP.

The third study provides a detailed elucidation of the chemical structure of ethanol organosolv lignin (EOL) of *B. davidii* by NMR spectroscopy and compares the data to that of the native (untreated) lignin. Such research was needed to understand the pretreatment mechanism in the context of delignification and alteration of the lignin structure. Future applications of the resulted EOL will be valuable for industrially viable bioethanol production process. EOP mainly cleaved *β-O*-4' interlinkages via homolysis, decreased the DP of lignin, and increased the degree of condensation of lignin. EOL had low oxygen content, molecular weight, and aliphatic OH as well as high phenolic OH, which are qualities that make it suitable for different co-product applications.

The last study provides information on the anatomical characteristics of pretreated *B. davidii* biomass after EOP. The importance of this research was to further understand

the alterations that occur to the cellular structure of the biomass which can then be correlated with its enzymatic digestibility. The results concluded that the physical distribution of lignin within the biomass matrix after pretreatment, and the partial removal of middle lamella lignin were key factors influencing enzymatic hydrolysis.

CHAPTER 1

INTRODUCTION

Finding an alternative source of energy to fossil fuels is undoubtedly one of the most important necessities and challenges that our society is currently addressing. The urgency to develop new energy production technologies emerges from the growing global energy demand and concerns about the negative effects of increasing greenhouse gas emissions from fossil fuels [1-4]. Therefore, the new form of energy must be sustainable, renewable, environmentally friendly, and economically/commercially feasible. Biofuel from lignocellulosic biomass is a compelling candidate for alternative fuel production because such materials are readily available, avoid issues surrounding 'food or fuel', and have the potential of having a relatively small environmental impact [5]. Biorecources such as hybrid poplar, switchgrass, *Miscanthus*, southern pine, willow, and corn stover are few species that have emerged as front-runners in the field of biofuels [6]. However, new biomass species should be explored in order to expand this bioresource base. In this dissertation (Chapter 4), a novel lignocellulosic material (*Buddleja davidii*) was characterized to determine the possibility of being a new feedstock for biofuel production.

Although the cost of biomass is low, releasing fermentable sugars from these materials remains challenging. The biochemical process of converting biomass to bioethanol consists of three main steps: pretreatment, enzymatic hydrolysis, and fermentation [7]. The pretreatment stage is generally considered to be the most costly process and has a significant impact on the efficiency of enzymatic hydrolysis and

subsequent fermentation [8-10]. The pretreatment stage is required to reduce the recalcitrance of lignocelluloses and remains the most intensive operating/operating cost component of cellulosic ethanol production [8, 11]. For this reason, researchers have focused on optimizing pretreatment conditions as well as understanding the fundamental characteristics of the biomass after pretreatment. Lignocellulosic biomass constitutes three biopolymers: cellulose, hemicellulose, and lignin, which together form a complex and rigid structure [1, 12]. This structure causes the biomass to be recalcitrant to biological and chemical degradation [12].

Improving our fundamental knowledge of pretreatment technologies will lead to significant advances in the field of sustainable low-cost cellulosic biofuel production [8]. Chapters 5, 6, and 7 address such necessities. The study in chapter 5 focuses on finding the ethanol organosolv pretreatment conditions for *B. davidii* that will provide efficient subsequent enzymatic hydrolysis of cellulose to glucose. The second part of the chapter examines the changes in the structure of cellulose after pretreatment. Chapter 6 describes the chemical transformations that occur to *B. davidii* lignin during pretreatment; while the last chapter provides information on the changes in the anatomical characteristics of *B. davidii* biomass after pretreatment.

The major objectives set in this dissertation are summarized as follows:

- Determine the basic composition of *B. davidii* as well as the structures of the plant cell wall biopolymers (cellulose and lignin).
- Define the positive and negative features of *B. davidii* from an agro-energy perspective.

- Perform different ethanol organosolv pretreatments on *B. davidii* and determine the composition of the pretreated biomass.
- Provide enzymatic hydrolysis profiles for various pretreated biomass.
- Characterize the fundamental chemical structure of cellulose and lignin after pretreatment by NMR spectroscopy and molecular weight analyses.
- Correlate between the enzymatic hydrolysis profile and the compositional analysis data as well as the chemical structure of cellulose and lignin.
- Explore the microscopic effects of ethanol organosolv pretreatment on *B. davidii* by determining the changes in the anatomical features of the biomass after pretreatment.

CHAPTER 2

LITERATURE REVIEW

2.1 Problem Statement

The demand for energy is increasing every year and is expected to grow by more than 50% by 2025, but the finite amount of fossil fuel available will be insufficient to satisfy this increase [1, 13]. Global petroleum demands have increased steadily from 63.1 x 10^6 barrels/day in 1987 to 84.0 x 10^6 barrels/day in 2010 (Figure 1) [14]. These demands and the recognition that society needs to develop sustainable energy technologies have focused the attention of many researchers on the development of alternative and renewable sources of energy, especially transportation fuels.

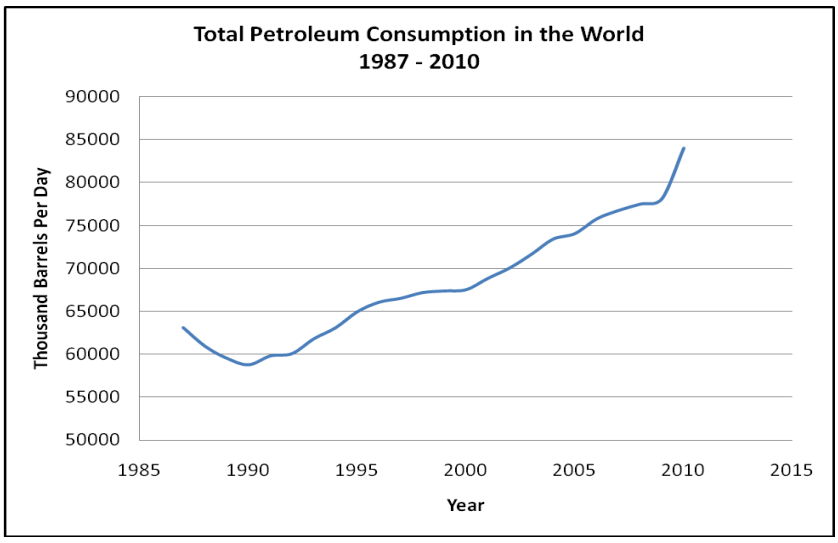


Figure 1. Global petroleum consumption from 1987 until 2010 [14].

The necessity to develop alternative transportation fuels has become a global concern mainly due to three factors: 1) increase in energy consumption, 2) finite

petroleum reserves, and 3) climate change [1, 15, 16]. It is believed that the production of bioethanol from lignocellulosic materials (biomass), such as wood, is one of the promising technologies in providing an alternative source of energy. These materials are renewable, abundant, and generate very low net CO_2 emissions. The use of biofuels improves national energy security, increases economic growth, and provides environmental benefits [17]. Biofuels production will decrease dependence on foreign sources of energy, reduce petroleum trade deficit, and reduce greenhouse gas (GHG) emissions [17]. The biofuels industry will lead to new economic development jobs and strengthen the agricultural markets [17].

Unlike the combustion of fossil fuels which releases CO_2, CO_2 released during the utilization of biofuels is balanced by CO_2 captured in the recent growth of the biomass, resulting in far less net impact on greenhouse gas levels [17, 18]. For instance, the usage of cellulosic ethanol as a transportation fuel is expected to reduce greenhouse gas emissions significantly (by 86%) [17]. The use of E10 in gasoline vehicles (GV) causes 85% reduction in GHG emissions, while E85, compatible with flexible-fuel vehicles (FFV), reduces GHG emissions by 86% [19]. The increase in the global CO_2 emissions from petroleum consumption made the necessity to develop alternative green energy a high priority (Figure 2) [14].

Furthermore, the increase in oil prices has led to the increase in interest in developing cheaper source for transportation fuel. The price of crude oil started to significantly increase around the year 2004, as indicated by Figure 3, and was at its prime in 2008 to reach 137 U.S. dollars per barrel [14]. Our increase dependence on and consumption of petroleum fuel will only drive the prices higher.

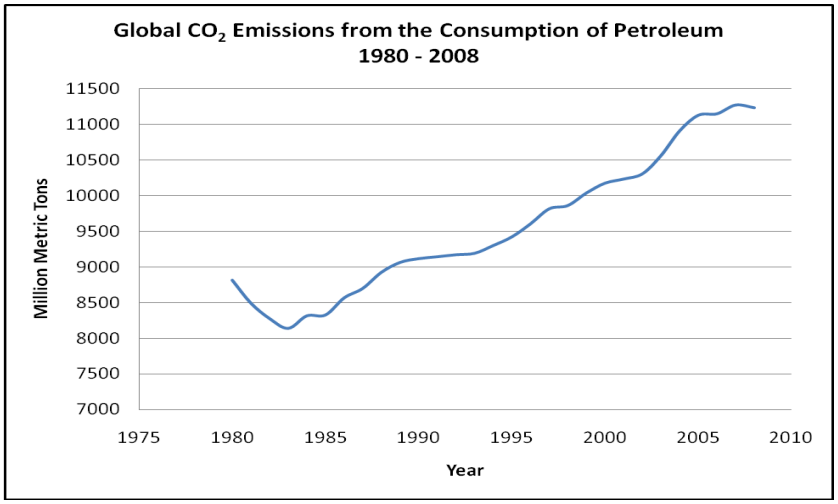


Figure 2. Global CO_2 emissions from the consumption of petroleum from 1980 to 2008 [14].

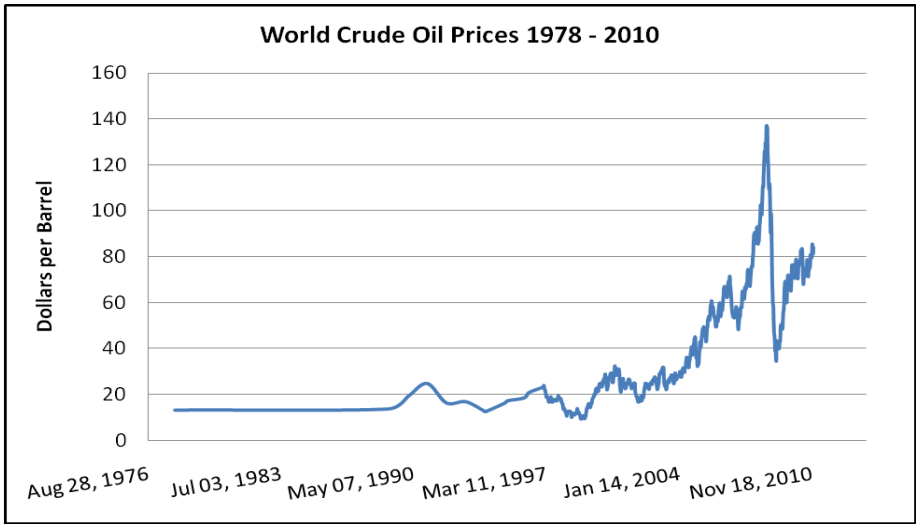


Figure 3. Data summary of the world crude oil prices from 1987 to 2010 [14].

2.2 Cellulosic Ethanol

Biofuels are a sustainable and renewable source of energy that has become of interest to many researchers over the past decade [2, 7, 8, 20]. The leading second-generation biofuel is cellulosic bioethanol in which a cellulosic biomass is converted to ethanol. First-generation biofuels are dependent on the fermentation of starches or sucrose derived from corn starch and sugar cane, respectively (Figure 4) [2, 13]. However, since these bioresources have food value and require productive agricultural lands, research focus has shifted toward bioethanol produced from lignocellulosic biomass [2]. Such materials do not directly compete with food, have a greater net energy generation per area of land, and can be grown on non-agricultural lands in a sustainable manner [18].

The bioconversion of lignocellulosic biomass to ethanol can be divided into a number of steps (Figure 4) [21]. Firstly, pretreatment must occur in order to improve accessibility of cellulose for the subsequent enzymatic hydrolysis (saccharification) by cellulases. This extra pretreatment step that is required in second-generation biofuels (based on lignocellulosic biomass) as opposed to first-generation biofuels (based on food commodities such as starch and sugar) makes the bioconversion process more energy and capital intensive [21]. After hydrolysis, the next step is the fermentation of glucose to ethanol, and finally, distillation [18].

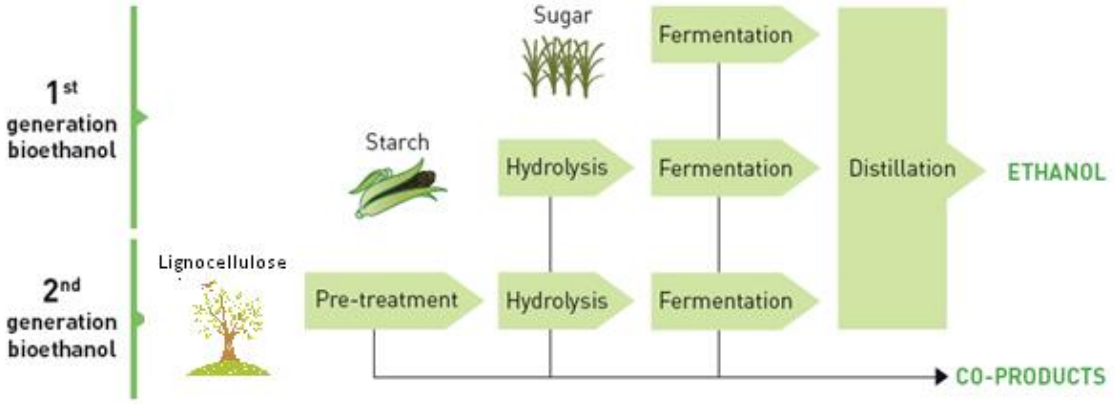


Figure 4. An overview of the bioconversion of lignocellulosic biomass to ethanol: 1st and 2nd generation bioethanol [21].

2.2.1 Bioresources

Biomass is an abundant resource with an annual global production rate of 170 x 10^9 metric tons [22]. A recent report suggested that more than 1.3 billion tons of biomass could be produced every year in the U.S. on a sustainable basis that would derive mostly from agricultural and forestry sources [16]. There is a wide variety of biomass crops that are being considered for widespread growth for biofuel applications. These include grasses such as *Miscanthus* × *giganteus* and switchgrass, as well as woody species such as poplar, willow, sweetgum, eucalyptus, and pine [18]. The choice of crop for biomass production depends largely on the growth conditions of a particular region. To obtain enough biomass for large-scale production of biofuels, a variety of agricultural and woody crops will need to be grown in different geographies and regional climates specifically suited for each bioresource. Table 1 summarizes the annual yield of some biomass currently being investigated for biofuel production.

Table 1. Average productivity of several biomass species in the United States [6, 23].

Species	Average productivity (Mg ha^{-1} $year^{-1}$)
Poplar	5-11
Miscanthus	15-40
Switchgrass	14
Corn stover	8
Wheatstraw	6

To be able to cover our global energy needs, the chosen renewable resources must have high energy content, be available in high volumes, and be easily accessible at a low cost. Such requirements are crucial so that the economical viability of the proposed renewable resource is valid [1].

2.2.1.1 *Buddleja davidii*

Buddleja davidii is an ornamental shrub that was introduced from China over 100 years ago, and has been naturalized in different parts of the world, including parts of the U.S. (Figure 5) and Europe [24]. *B. davidii* was discovered in 1880s by French missionaries in China, where it is known as the Summer Lilac [25]. The genus is named in honor of Reverend Adam Buddle (1660-1715), English botanist and vicar of Farmbridge in Essex [25]. The species is named after the French missionary and naturalist Father Armand David, who discovered it in 1869 [25].

This plant has several attractive agro-energy features that make it an interesting potential source for biofuels. *B. davidii* exhibits a very wide range of growth habitat and is well adapted to growing in any soil conditions [26]. Moreover, the plant is perennial, has favorable growth dimensions (up to 3-5 m high), and has very few pests or diseases [26].

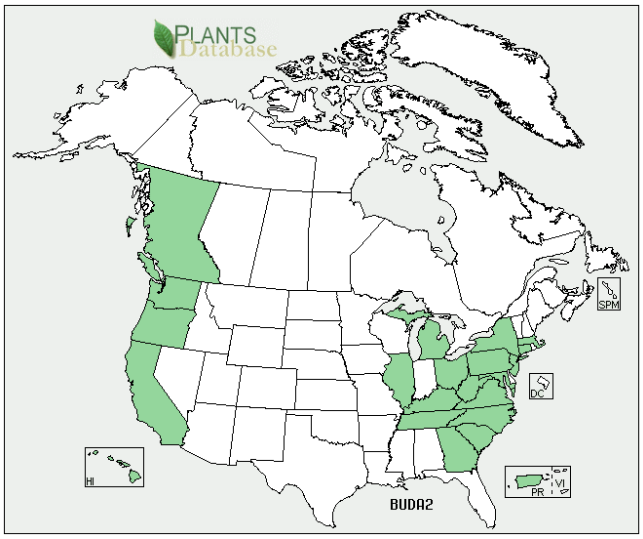


Figure 5. Distribution of *Buddleja davidii* in the U.S [27].

B. davidii has a high biomass yield, about 10-15 dry tons per hectare per annum. [25]. Such yield is comparable to that of other bioenergy crops such as hybrid poplar with annual yield of 7.9-11.8 dry tons per hectare and switchgrass with 14 dry tons per hectare per annum, but higher than corn stover with 8.4 dry tons per hectare per annum and wheatstraw with 6 dry tons per hectare per annum [6].

B. davidii has the ability to tolerate a wide spectrum of climatic conditions including oceanic, continental and Mediterranean [25]. In Europe, *B. davidii* is found growing in all soil types (Figure 6). In low land areas, it favors drier mineral soils. When planted it can and will tolerate almost any soil type but does not appear to be able to grow in very wet areas. It has been found to grow well in soils with pH values between 5.5 and 8.5 [25]. *B. davidii* is tolerant to drought and can survive growing in walls and rock faces and still attain a large size within such limited environs. It favors heavily disturbed areas such as those found in quarries and railway sidings and is a frequent member of plant communities in urban wastelands [25]. *B. davidii* has been described as an ecological

specialist [28] because of its ability to grow on new, coarse-textured, nitrogen deficient land by its drought-tolerant seedlings which quickly develop into an extensive root system to tap distant water [29]. Their extensive root systems reduce erosion and increase soil quality. It prefers open areas but will tolerate some light shade. It is unable to survive the harsh winters of northern continental climates, being killed by temperatures below about -15 °C to -20 °C [25].

Figure 6. Images of *Buddleja davidii* that illustrate its growth habitat. a, vigorous growth in open and poor soil. b, growth in very poor sites. c, growth in no soil at all.

Being a perennial plant, *B. davidii* provides several advantages. Perennials are "low-maintenance" crops because they are not planted annually like most traditional row crops [30]. Also they have longer growing seasons and require little fertilizer and other inputs [30]. In addition, a large fraction of mineral nutrients from above-ground portions of the perennial plants are deposited underground before harvest [30].

B. davidii's foliage and flowers contain flavonoids, triterpenes and carotenoids which have anti-inflammatory properties, flavonoids and phenylethanoids which are antioxidant and undetermined substances which might cause proliferation of fibroblasts (Figure 7) [31]. In addition, antibacterial compounds have been found such as verbascoside which acts against *Staphylococcus aureus* [3]. *B. davidii* has also shown to

have antifungal activity [32]. Chloroform extracts of *B. davidii* bark have been found to be effective against the soil fungi *Fusarium colmorum* and *Sordari fimicola* [32]. The major compound responsible for this activity was shown to be buddledin A (Figure 7). As society moves toward the concept of biorefinery, this plant may well be cultivated for value added chemicals, and the plant biomass residue could then be a viable resource for biofuel production.

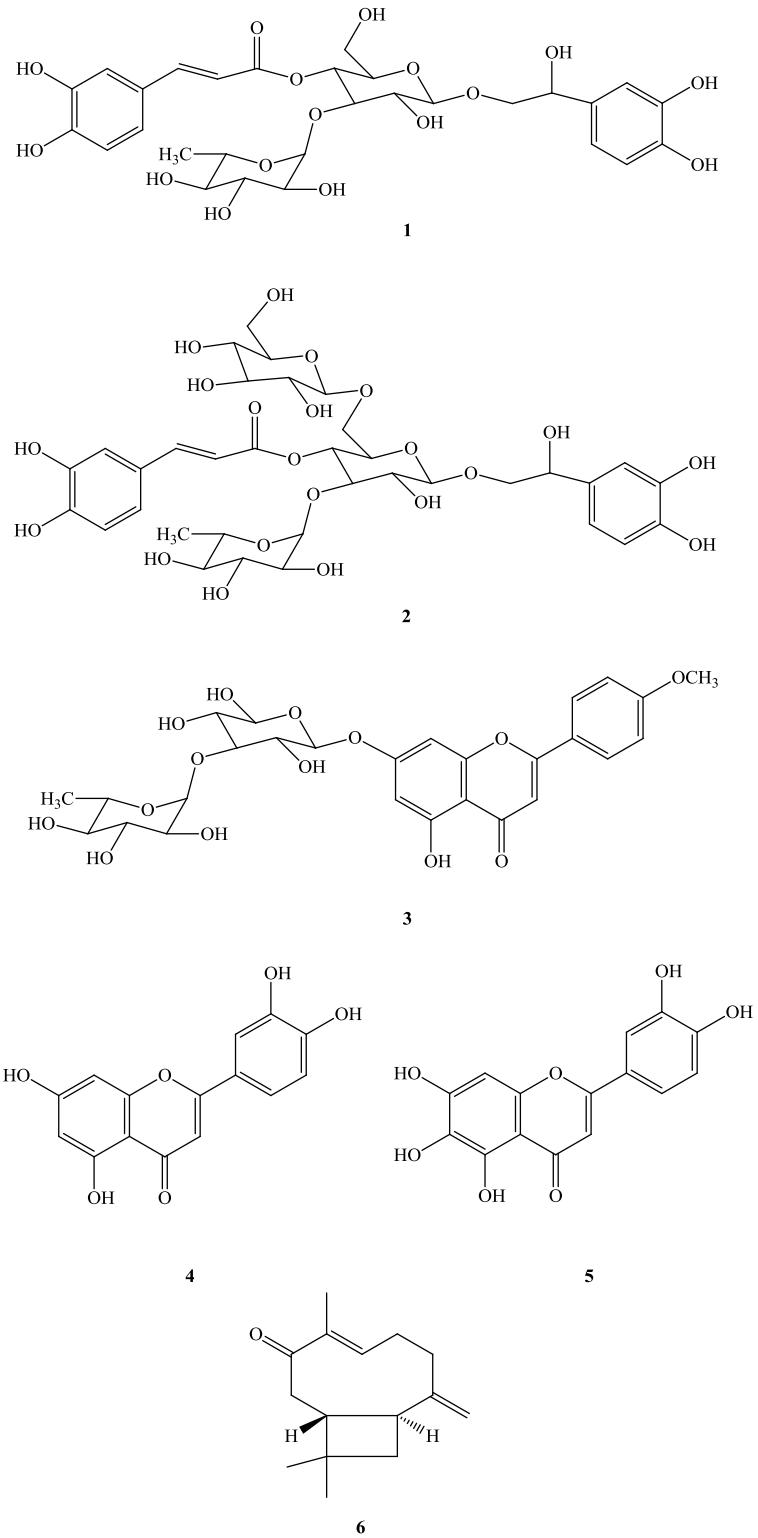


Figure 7. Examples of some compounds isolated from *Buddleja* that have anti-inflammatory and antifungal activity. **1** and **2** are a trisaccharide and disaccharide, respectively with phenylethanoid and caffeic acid attachments; **3**, **4**, and **5** are flavonoids; and **6** is buddledin A [33, 34].

2.2.2 Lignocelluloses

Lignocellulosic biomass is composed of three major constituents: cellulose, hemicelluloses and lignin (Figure 8), as well as smaller amounts of pectin, protein, extractives and ash, which together form a complex and rigid structure [20, 35]. This structure causes the biomass to be recalcitrant to biological and chemical degradation [12]. Table 2 summarizes the distribution of the three major biopolymers in several hardwood, softwood, and agricultural residue species [3].

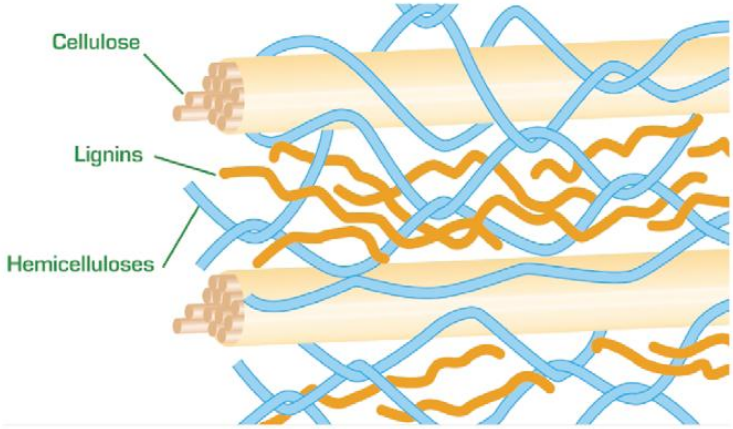


Figure 8. Schematic representation of the secondary cell wall containing cellulose, hemicelluloses and lignin [36].

Table 2. Cell wall macromolecule composition of different softwood and hardwood species [3, 37].

	Wood Macromolecules[a]		
Wood Species	**Cellulose (%)**	**Lignin (%)**	**Hemicelluloses (%)**
Softwoods			
Picea glauca	41	27	31
Abies balsamea	42	29	27
Pinus strobes	41	29	27
Tsuga canadensis	41	33	23
Norway spruce	46	28	25
Loblolly pine	39	31	25
Thuja occidentalis	41	31	26
Hardwoods			
Eucalyptus globulus	45	19	35
Acer rubrum	45	24	29
Ulmus americana	51	24	23
Populus tremuloides	48	21	27
Betula papyrifera	42	19	38
Fagus grandifolia	45	22	29
Agricultural residues			
Corn stover	40	25	17
Wheat straw	30	50	20
Switchgrass	45	30	12

[a]all samples were analyzed extractives free

Cell wall in biomass is composed mainly of middle lamella (ML), primary cell wall (P), and secondary cell wall (S) (Figure 9) [38]. ML is an amorphous intercellular region, containing mostly lignin that glues the fibers together. The primary cell wall is a thin layer (0.1 – 1 μm) contains of a loose, random network of microfibrils (cellulose) embedded in a matrix that consists of amorphous pectins and hemicelluloses lacking structural orientations [39]. The secondary cell wall is thicker than the primary wall (10 – 20 μm) and is divided into three sublayers due to differences in the microfibrillar orientation. These layers are: the outer layer of the secondary wall (S1), the middle layer of the secondary wall (S2), and the inner layer of the secondary wall (S3). The S1 layer contains fibrils that are arranged in a cross-hatch pattern; the S2 accounts for a major part

of the cell wall volume and is composed of parallel fibrillar units oriented at a slight angle to the cell axis; the S3 contains parallel fibrillar units forming a flat helix in the transverse direction [39].

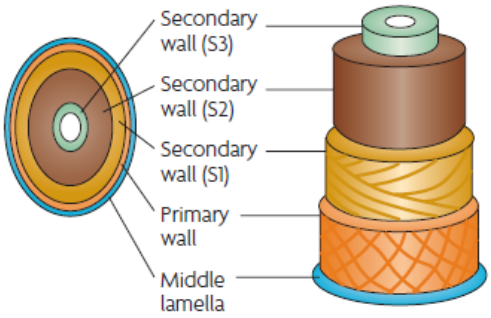


Figure 9. Schematic representation of plant cell wall [38].

2.2.2.1 Lignin

The term lignin is derived from the Latin word "lignum," for wood [40, 41]. Lignin is an amorphous, cross-linked, and three dimensional phenolic polymer consisting of methoxylated phenylpropane structures [2]. A schematic representation of a proposed softwood and hardwood lignin structures are depicted in Figure 10 and Figure 11, respectively [42, 43]. Lignin is considered to be the most recalcitrant biopolymer in the plant cell wall and provide three main functions. First, lignin decreases the permeability of water across the cell walls, which is an important role for the transport of water and nutrients [41]. Second, it provides rigidity and structural support to the cell wall to resist compression and bending [41]. Third, lignin can protect the cell wall from microorganisms by providing resistance against the penetration of destructive enzymes into the cell wall [41].

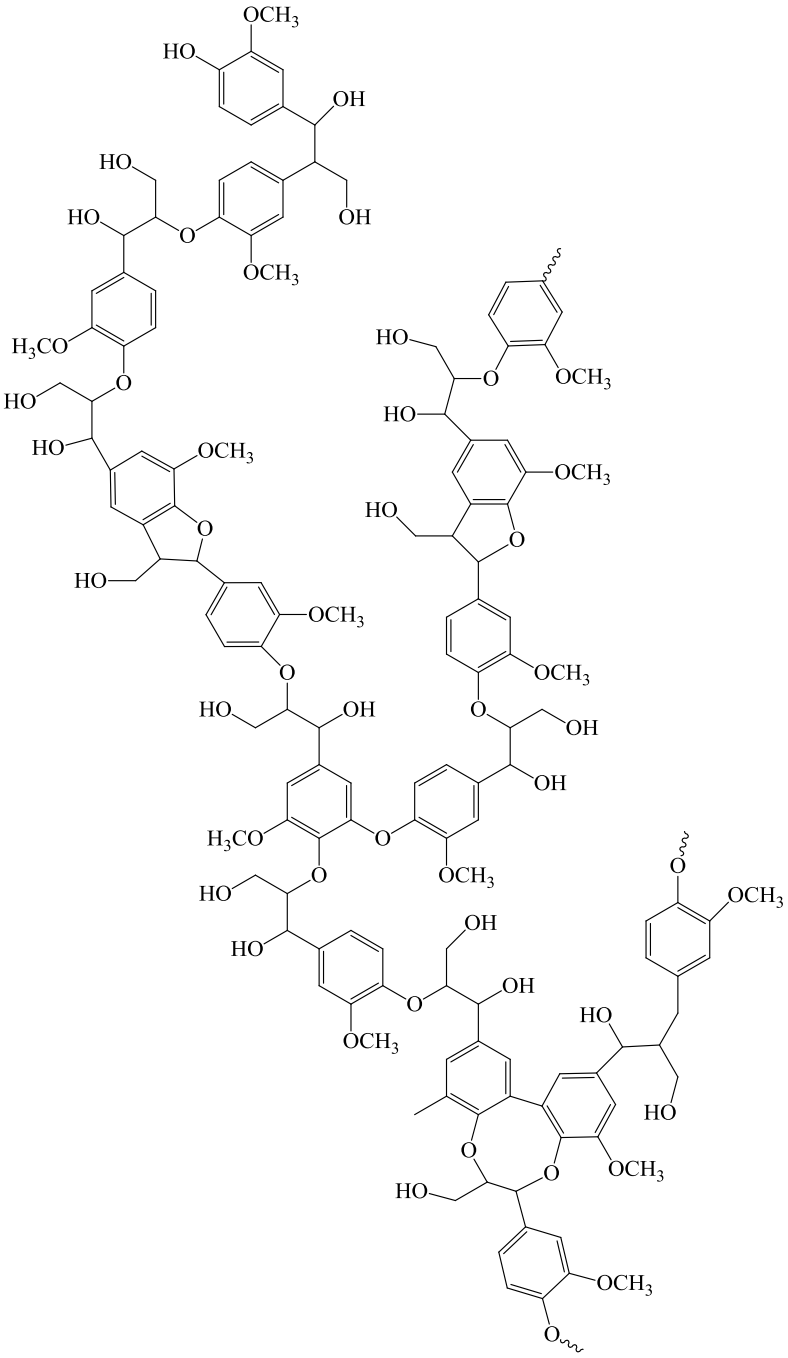


Figure 10. Example for structure of native softwood lignin [42].

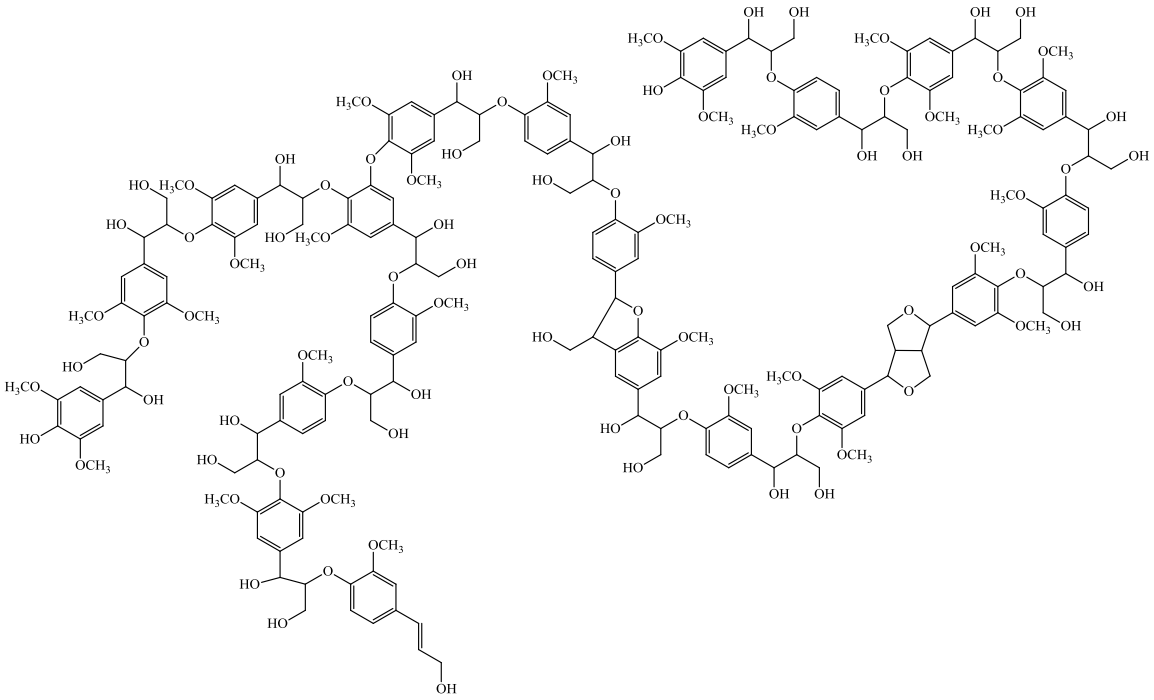


Figure 11. Model for poplar milled wood lignin [43].

Lignin can be biosynthesized from three monolignols: coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol (Figure 12) [2, 44]. The polymerization process is initiated by the oxidation of the monolignol phenolic hydroxyl groups. The oxidation itself has been shown to be catalyzed via an enzymatic route [2, 44]. It is believed that both peroxidases and laccases are involved in lignin synthesis, where laccase is primarily responsible for the initial polymerization of monolignols to oligolignols, while peroxidases, on the other hand, catalyze the reactions of oligolignols leading to the extended lignin macropolymer [45]. The enzymatic dehydrogenation is initiated by an electron transfer that yields reactive monolignol species with free radicals (Figure 12). A monolignol with a free radical can then couple with another monolignol with a free radical to generate a dilignol. Subsequent nucleophilic attack by water, alcohols, or phenolic hydroxyl groups on the benzyl carbon of the quinone methide intermediate will

restore the aromaticity of the benzene ring [2]. The generated dilignols will then undergo further polymerization. Interunit linkages include: *β*-*O*-aryl ether (*β*-*O*-4'), resinol (*β*-*β'*), phenylcoumaran (*β*-5'), biphenyl (5-5'), and 1,2-diaryl propane (*β*-1') (Figure 12) [2, 46]. An example of a radical coupling is shown in Equation 1 [40]. Typical proportions of lignin linkages in softwood and hardwood species are summarized in Table 3. Softwood lignin is composed mainly of guaiacyl units and trace amounts of *p*-hydroxyphenyl units, while hardwood lignin comprises of guaiacyl (G) and syringyl (S) units with minor amounts of *p*-hydroxyphenyl (H) units. Grass lignin typically contains significant amounts of all three of the lignin units. Table 4 summarizes typical H:G:S ratio for lignin from biomass.

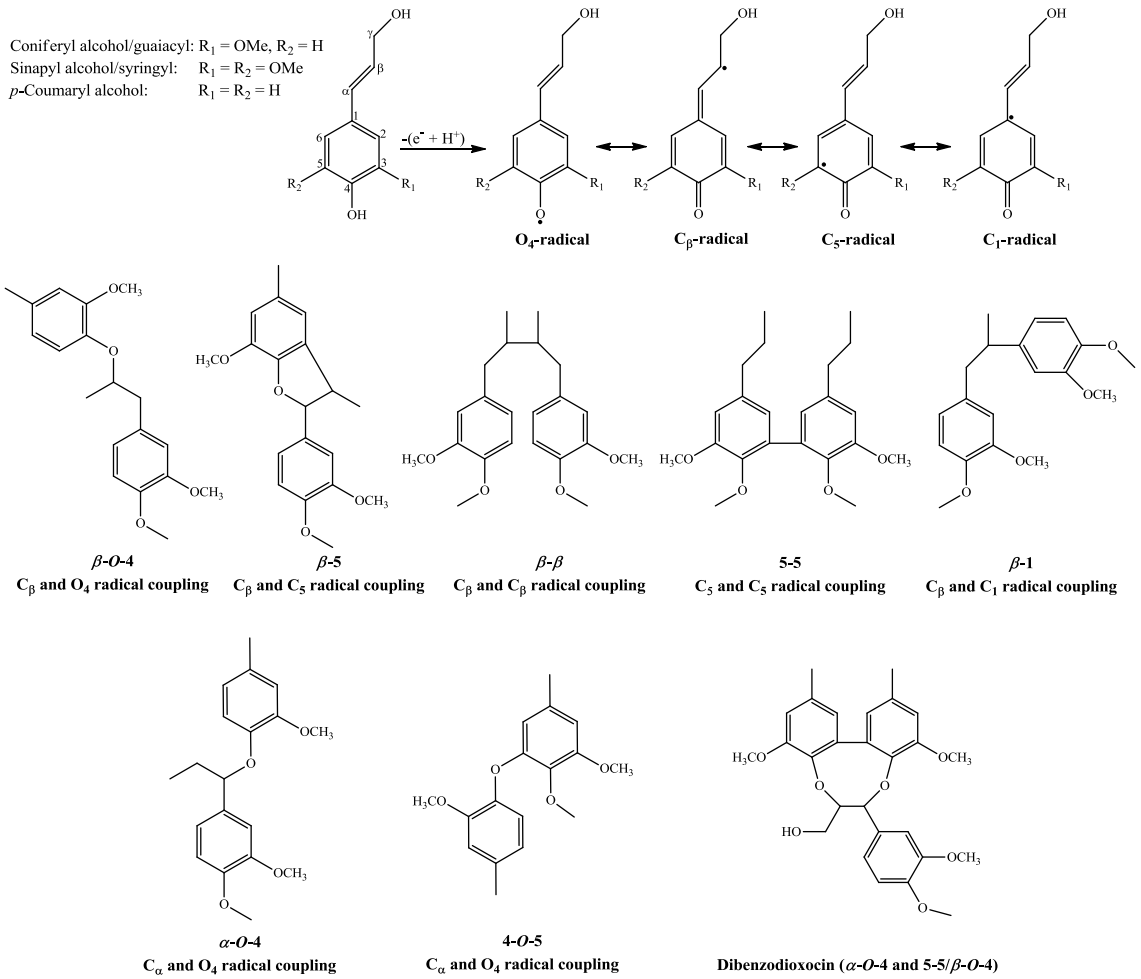
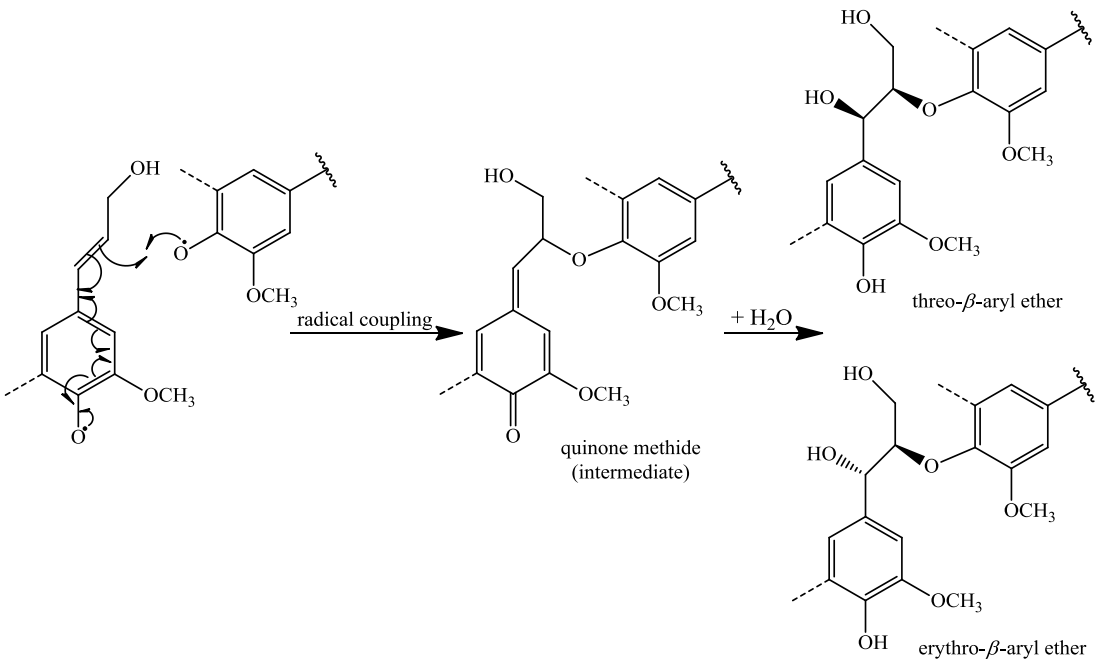


Figure 12. Some common linkages found in lignin formed via radical coupling between phenylpropane units [2, 46].



Equation 1. Formation of *β*-O-4 interlinkage via radical coupling [40].

Table 3. Proportions of different types of lignin interlinkages per 100 Aromatic units in softwood and hardwood lignin.

Linkage type	Spruce lignin [47]	*Eucalyptus grandis* lignin [48]
β-O-4'	45	61
α-O-4'	16	n.d.
β-β′	2	3
β-5'	9	3
5-5'	24-27	3
β-1'	1	2
4-*O*-5'	n.d.	9
Dibenzodioxocin	7	n.d.

Table 4. Molar percentage of guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units in several biomass lignin.

Origin	G	S	H
Wheat straw [49]	45	46	9
Loblolly pine [50]	87	0	13
Spruce [47]	98	2	0
Beech [51]	56	40	4
Eucalyptus globulus [52]	14	84	2
Alamo Switchgrass [11]	51	41	8

In addition, lignin and carbohydrates are covalently bound to form lignin-carbohydrate complexes (LCC). In wood, LCCs are covalently bound by benzyl ester and benzyl ether linkages, through sugar hydroxyl to the α-carbanol of phenylpropane subunits in lignin, as well phenylglucosidic linkages [53, 54]. Figure 13 and Figure 14 represents possible ester and ether linkages in LCC [53].

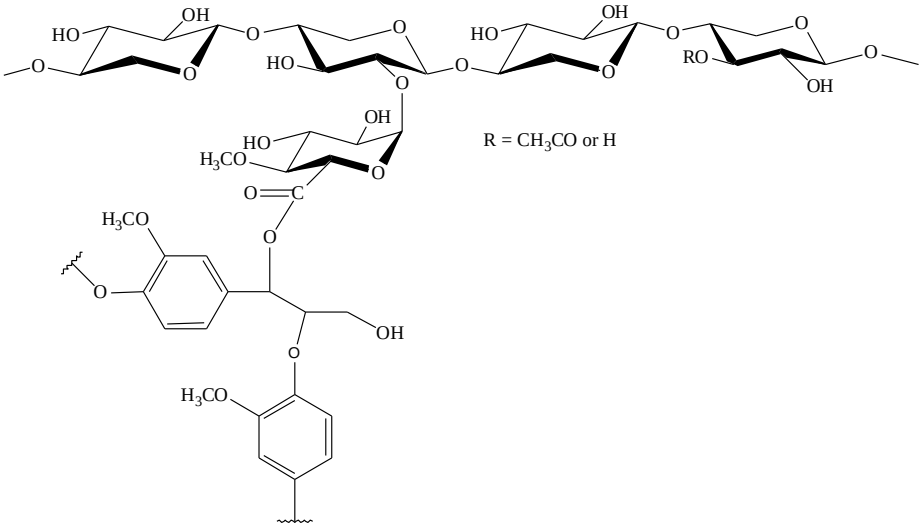


Figure 13. Structure of ester linkage between lignin and glucuronoxylan [53].

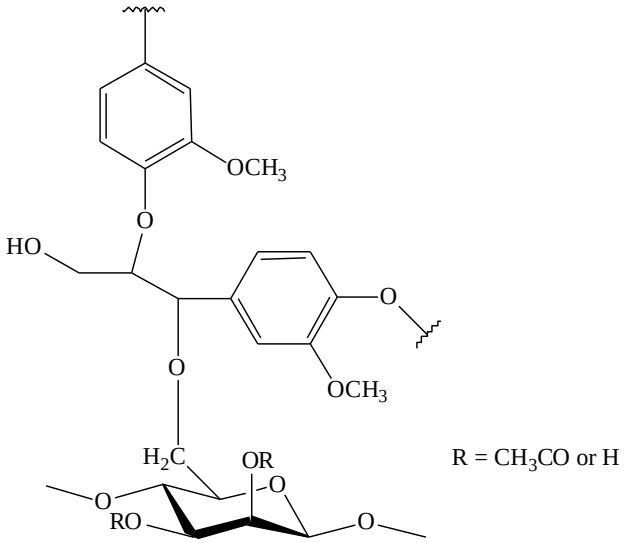


Figure 14. Structure of ether linkage between lignin and glucomannan [53, 55].

Lignin can covalently bind to cellulose, xylan, glucomannan, and galactan. For instance, analysis of spruce LCCs showed that it contains the following structures: glucose-lignin (Glu-L) LCC, galactose-glucose-mannose-lignin (GalGlcMan-L) LCC, and glucose-mannose-lignin-xylose (GlcMan-L-Xyl) LCC. The compositions of these fractions are summarized in Table 5 [56].

Table 5. Sugar and lignin analysis of spruce LCC fractions [56].

	Sugar and lignin composition (% w/w)					
LCC fraction	Arabinan	Xylan	Mannan	Galactan	Glucan	Klason lignin
Glu-L	1.0	0.9	2.5	0.2	85.2	7
GalGlcMan-L	3.2	2.4	33.5	12.6	5.2	39
GlcMan-L-Xyl	1.2	3.1	19.2	4.0	9.3	56

In another study, lignin-carbohydrate complexes (LCCs) were isolated from unbleached spruce sulfite pulp [55]. The results showed that approximately 80% of the lignin in the pulp chemically linked (covalently bonded) to carbohydrates in three major fractions: one glucan-lignin complex (with 8% lignin), and two hemicellulose-lignin

complexes (with 72% lignin), which were one glucomannan-lignin complex (with ~25% lignin) and one xylan-lignin-glucomannan complex (with 45-50% lignin). The lignin was more covalently bound to hemicelluloses than cellulose.

2.2.2.2 Hemicelluloses

Hemicelluloces are polysaccharides with DP values ranging between 50-300 [2]. These polymers constitute of varying ratios of arabinose, xylose, galactose, mannose, and glucose, along with side chains groups, such as acetyl, galacturonic acid, glucuronic acid, and 4-O-methylglucuronic acid. These polymers are essentially amorphous. The major hemicelluloses in softwoods (SW) are galactoglucomannans and arabinoglucuronoxylan, while the predominant hemicellulose in hardwoods (HW) is glucuronoxylan (Figure 15) [2].

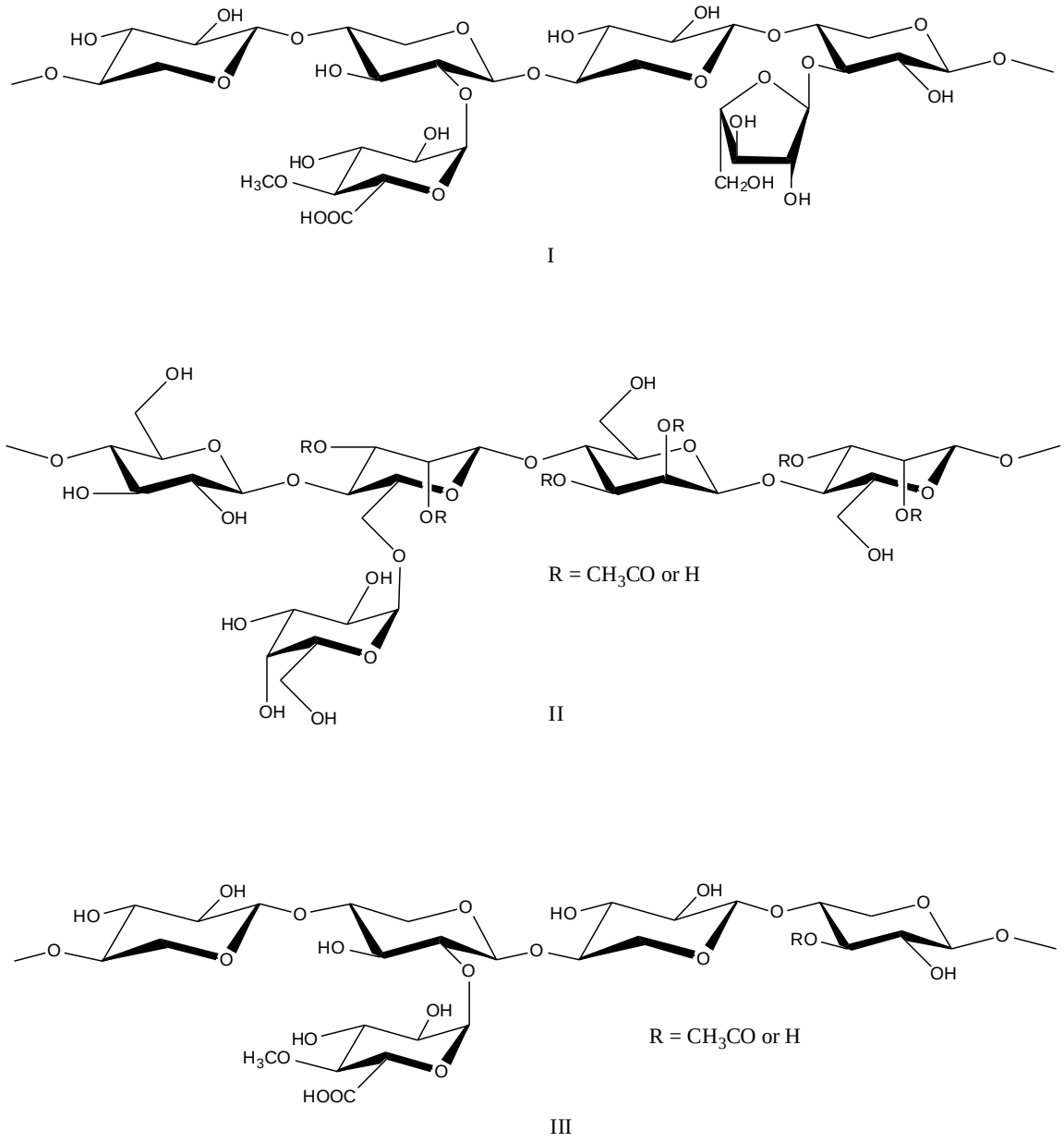


Figure 15. Principal structure of: (I) arabinoglucuronoxylan in softwood, (II) galactoglucomannans in softwood, and (III) glucuronoxylan in harwood [2].

Table 6 summarizes the main structural features of hemicelluloses appearing in common softwood and hardwood resources [2, 3, 57, 58]. Hardwood glucuronoxylan and softwood arabinoglucuronoxylan both have a backbone of (1→4)-linked β-D-xylopyranosyl units, but exhibit differences in branching and substitution patterns [42]. In the former, the C_2-OH and C_3-OH are partially acetylated (i.e., 3.5 – 7.0 acetyl groups/10 xylose) and are lesser amounts of (1→2)-linked pyranoid 4-O-methyl-α-D-glucuronic acid units [42]. For softwoods, the xylan polymer is not acetylated and typically is branched with (1→2)-linked pyranoid 4-O-methyl-α-D-glucuronic acid and (1→3)-linked α-L-arabinofuranosyl units with an arabinose:uronic acid:xylose ratio of ~1:2:8 [42]. Galactoglucomannan is comprised of (1→4)-linked β-D-glucopyranosyl and D-mannopyranosyl units that are partially acetylated at the C_2-OH and C_3-OH and partly substituted by (1→6)-linked α-D-galactopyranosyl units [42]. Softwoods generally have two different types of galactoglucomannans: one being highly branched with a ~1:1:3 ratio of galactose:glucose:mannose and another that is less branched with a ~0.1:1:3 ratio galactose:glucose:mannose [42]. In hardwoods, the glucomannan polymer has little or no branching with a typical glucose:mannose ratio of ~1:1.5 [42]. Table 7 summarizes hemicelluloses composition and its DP in several hardwood and softwood species [59].

Table 6. The major hemicelluloses components in softwood and hardwood [2, 3, 57, 58].

Wood	Hemicellulose type	Composition				~DP
		Amount (% of wood)	Units	Molar ratios	Linkage	
SW	Galactoglucomannan	10-15	*β*-D-Man*p*	4	1→4	100
			β-D-Glc*p*	1	1→4	
			β-D-Gal*p*	0.1	1→6	
			Acetyl	1		
	Arabinoglucuronoxylan	7-10	*β*-D-Xyl*p*	10	1→4	100
			4-O-Me-*α*-D-Glc*p*A	2	1→2	
			β-L-Ara*f*	1.3	1→3	
HW	Glucuronoxylan	15-30	*β*-D-Xyl*p*	10	1→4	200
			4-O-Me-*α*-D-Glc*p*A	1	1→2	
			Acetyl	7		
	Glucomannan	2-5	*β*-D-Man*p*	1-2	1→4	200
			β-D-Glc*p*	1	1→4	

Table 7. Hemicellulose composition and DP in several biomass [59].

Origin	Hemicellulose	Carbohydrates composition of the hemicellulose		DP
		Sugar residues	Molar ratio	
Birch	Glucuronoxylan	4-O-MeGlcA:Xyl	5:100	101-122
Aspen	Glucuronoxylan	4-O-MeGlcA:Xyl	9:100	101-122
Spruce	Arabinoglucuronoxylan	Ara: 4-O-MeGlcA:Xyl	6:13:100	107-145
	Galactoglucomannan	Gal:Glu:Man	16:24:100	118-132
Pine	Arabinoglucuronoxylan	Ara: 4-O-MeGlcA:Xyl	10:16:100	107-145
	Galactoglucomannan	Gal:Glu:Man	9:22:100	118-132
Larch	Arabinoglucuronoxylan	Ara: 4-O-MeGlcA:Xyl	10:12:100	107-145
	Galactoglucomannan	Gal:Glu:Man	8:26:100	118-132

2.2.2.3 Cellulose

Cellulose was first discovered by the French scientist Anselme Payen in 1838, when he noticed a resistant fibrous solid that remained behind after treating plant tissue with acids and ammonia. Cellulose is a linear polymer made up of β-D glucopyranose

units covalently linked with 1→4 glycosidic bonds, with the DP varying with the origin and treatment of the raw material (Figure 16) [60].

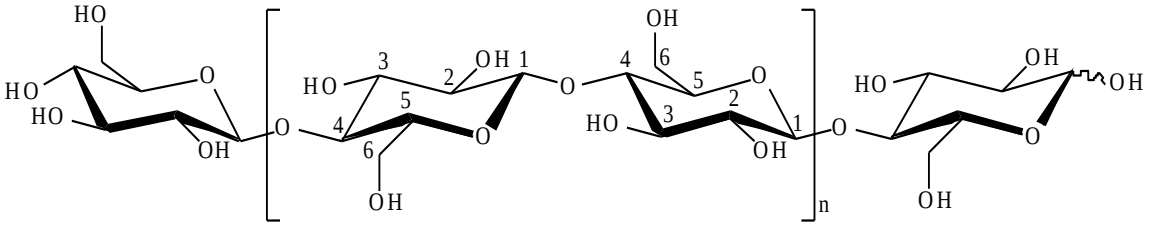


Figure 16. Molecular structure of cellulose.

The large number of hydroxyl groups on the cellulose chain forms intra- and inter-molecular hydrogen bonds, resulting in the crystalline structure of cellulose [2]. Native cellulose in plants is a composite of three crystalline allomorphs: cellulose I_{α}, cellulose I_{β}, and *para*-crystalline cellulose; and two noncrystalline forms: amorphous cellulose at accessible and inaccessible fibril surfaces [61-64]. Solid-state cross polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectroscopy is a technique that has been used to determine the relative intensities of these various cellulose structures as well as cellulose crystallinity. Table 8 summarizes the cellulose crystallinity and ultrastructure from several biomass determined by CP/MAS ^{13}C NMR spectroscopy [6]. Cellulose I_{α}, a one chain triclinic unit cell, is the dominant form in bacterial and algal cellulose, whereas cellulose I_{β}, a monoclinic two-chain unit cell, is dominant in higher plants, such as wood [64]. *para*-Crystalline cellulose is the form that is less ordered than cellulose I_{α} and cellulose I_{β} but more ordered than amorphous cellulose [64]. Accessible fibril surfaces are those in contact with water, while the inaccessible fibril surfaces are fibril-fibril contact surfaces and surfaces resulting from distortions in the fibril interior

[65]. Cellulose I_α is meta-stable in nature and can be converted to the thermodynamically more stable allomorph (cellulose I_β) by annealing [64, 66]. Nishiyama *et al*. proposed that slippage of the glucan chains is the most likely mechanism for conversion of cellulose I_α to cellulose I_β [66]. Both chains in cellulose I_α and I_β are organized in sheets packed in a parallel-up fashion, with the hydroxymethyl groups in the *tg* conformation [66].

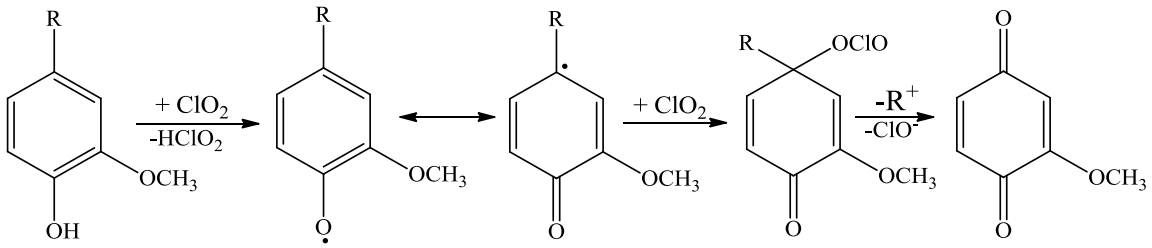
Table 8. Cellulose crystallinity and ultrastructure from several biomass determined by CP/MAS ^{13}C NMR spectroscopy [6].

Origin	Crystallinity (%)	I_α	$I_{\alpha+\beta}$	*para*-Crystalline	I_β	Accessible fibril surfaces	Inaccessible fibril surfaces
Hybrid poplar	63	5.0	14.2	31.1	19.8	10.2	18.3
Loblolly pine	63	0.1	30.7	24.8	6.9	33.1	15.6
Alamo switchgrass	44	2.3	8.8	27.3	4.5	5.7	51.3

2.2.2.3.1 Isolation of Cellulose for DP Measurements

In order to analyze DP of cellulose, it must first be purified and isolated from its native source (i.e. separated from the extractives, lignin, and hemicelluloses). The common method for the isolation of cellulose is that described by Browning, as this method does not alter the chain length of cellulose significantly [67]. First, the air-dry wood meal is pre-extracted with 95% ethanol in a Soxhlet extractor. Then the wood is delignified by using glacial acetic acid and sodium chlorite, which results in the formation of holocellulose (i.e. a mixture of cellulose and hemicellulose) by a selective oxidative degradation of lignin. Typically, four-hour holocellulose pulping is sufficient, but this involves adding more CH_3COOH and $NaClO_2$ after the first, second, and third hour. The reaction temperature is usually between 70-80 °C and yields a relatively pure

cellulose-hemicellulose product. During the holocellulose pulping, the chlorite acts as the oxidant and reacts mainly with phenolic hydroxyl lignin as follows [68]:



Equation 2. Reaction mechanism of chlorine dioxide with phenolic hydroxyl lignin.

Lastly, the cellulose is isolated from holocellulose by extraction with concentrated sodium hydroxide according to the TAPPI T-203 cm-09 method, in which holocellulose is treated with 17.5% NaOH by weight at room temperature for about 1 hour to hydrolyze the hemicelluloses. The resulted cellulose is usually called α–cellulose. The chemical dosage, temperature, and period of reaction are factors that affect degradation of cellulose during the isolation process and should be adjusted depending on the species and their composition.

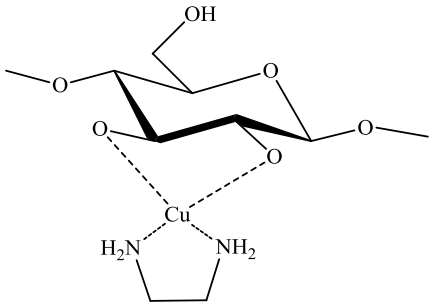
The acid-chlorite delignification method selectively removes lignin from biomass with only trace solubilization of glucan and hemicelluloses, such as xylan [69]. Recently, there has been a concern about this method that it may significantly reduce the chain length of cellulose. Kumar *et al*. reported a reduction of nearly 75% in the viscosity average degree of polymerization (DP_V) of filter paper cellulose after delignification [70]. However, it has been noted that an important consideration for this method is to allow a few percent of lignin to remain in the preparation, because complete delignification will result in excessive loss of polysaccharides [67]. Furthermore, Hubbell and Ragauskas

have recently reported that lignin-free filter paper samples showed a DP reduction of nearly 35% after holocellulose delignification, while a reduction of 12% was observed for samples that contained 30% lignin, and 17% when lignin content reached 1% [71]. Thus, small amount of lignin (~1%) can minimize loss of cellulose DP during holocellulose pulping.

2.2.2.3.2 Determining Cellulose Degree of Polymerization

The acid-chlorite delignification followed by alkali treatment technique for cellulose isolation from lignocellulosic material has been used by several studies prior to measuring cellulose DP [70, 72-76]. Cellulose has to be purified or isolated from its native source in order to adequately determine its DP [77, 78]. The presence of hemicelluloses and lignin would alter the average DP value of cellulose. The two most commonly used techniques to measure the DP of cellulose are the viscometry and the gel-permeation chromatography (GPC) methods. The determination of the DP of cellulose begins with its dissolution using a method that does not significantly affect its original DP. Cellulose is insoluble in all known common solvents because of its high molecular weight, intra- and intermolecular hydrogen bonding, and the resulting high crystallinity [79]. The two most practiced techniques to dissolve cellulose are: 1) dissolving cellulose in metal complex solutions or 2) forming cellulose derivatives by nitration or tricarbanilation. Metal complex solutions such as Cuam and Cuen have been used as medium to dissolve cellulose. The Cuam solution is a copper complex with ammonia, and Cuen solution is copper complex with ethylenediamine that is also known as cupriethylenediamine [80]. These solutions have been used for measuring the DP of cellulose either viscosimetrically or by using GPC [78, 80]; however, the

cupriethylenediamine solution has been frequently used with the viscosimetric technique [70, 72-74, 76, 81-83]. The solubility of cellulose is due to the formation of a cellulose-copper complex [84]. The complexes between cellulose and Cuam or Cuen, repectively, are formed by chelation of the glycol groups in the anhydroglucose base units with the copper displacing in each interaction two moles of ammonia or one mole of ethylenediamine from two of the four coordination sites [84]. This is illustrated in the following chemical formula [84]:



DP can be defined in terms of the number average (DP_N), weight average DP (DP_W), or viscosity average DP (DP_V), according to the following equations [85]:

$$DP_N = \frac{M_n}{MW_{glu}} = \frac{\sum N_i M_i}{\sum N_i} / MW_{glu}$$

$$DP_W = \frac{Mw}{MW_{glu}} = \frac{\sum N_i M_i^2}{\sum N_i} / MW_{glu}$$

$$DP_V = \frac{M_V}{MW_{glu}} = \frac{\sum N_i \eta}{\sum N_i} / MW_{glu} \text{ , where } \eta = K_m M_i^{\alpha+1}$$

where N_i is the number of moles of a given fraction *i* having molar mass M_i, M_N is the number-average molecular weight, M_W is the weight average molecular weight, M_V is the

viscosity-average molecular weight, MW_{glu} is the molecular weight of anhydroglucose (162 g/mol), η is viscosity, K_m is a constant, and the value of α for cellulose and cellulose derivatives in most cases ranges from 0.75 – 1 [85]. Viscometry measurements are relatively quick and convenient and are used to measure the DP_V of cellulose [86]. However, it has three limitations: 1) it provides only M_V, which is not an absolute average since it depends of the solvent/temperature conditions, 2) it provides no information concerning the molar mass distribution (MMD), and 3) the complex metal solutions used along with the method can degrade cellulose [86]. On the other hand, GPC provides DP_n, DP_w, all three molecular weights (M_N, M_W, and M_V), as well as the MMD. Similar to viscometry, GPC does not give the absolute molar mass because it is calculated based on the molecular weight of a set of standards which is frequently a set of well defined polystyrene standards with varying molecular weights. A certain range of the standards are used, depending on the nature of the analysis, in order to construct a calibration curve, which is then used to obtain the molecular weight of CTC. Despite the differences, researchers have used both methods to measure the DP of native woody and non-woody cellulose.

Measuring the DP of cellulose viscometrically after nitration was a technique developed in the 1940s, in which extractive-free wood meal is treated in a mixture of nitric acid, phosphoric acid, and phosphorous pentoxide, resulting in the isolation of cellulose nitrates, which is then solubilized in either acetone or ethyl acetate [87-89]. In the 1950s, T. E. Timell extensively investigated this technique and determined the DP of several native wood celluloses [88-90]. A list of the DP of cellulose determined by nitration followed by measurement of viscosity is provided in [87-91]. An advantage of

this method is that it does not require pre-isolation of cellulose through the holocellulose pulping and the base-catalyzed hydrolysis of the hemicellulose. The DP values ranged from ~925 – 5500. Table 9 shows that agricultural residues, such as bagasse and wheat straw, have lower cellulose DP (~1000) than hardwoods and softwoods, which possess higher DP cellulose in the range of 4000 – 5500.

Table 9. DP of native wood and non-woody celluloses after nitration using the viscometric method [87-91].

Species	DP
Trembling aspen	5000
Beech	4050
Red maple	4450
Eastern white cedar	4250
Eastern hemlock	3900
Jack pine	5000
Tamarack	4350
White spruce	4000
Balsam fir	4400
White birch	5500
Eucalyptus regnans	1510
Pinus radiata	3063
Bagasse	925
Wheat straw	1045

Today the nitration methodology is rarely used due, in part, to the uncertainty arising from possible acid hydrolysis of the cellulose chain during derivatization as well as the instability of the derivative [78]. The acid hydrolysis of cellulose by nitric and phosphoric acids is more dramatic when derivatizing pure cellulose (i.e. without the presence of lignin or hemicellulose). For instance, the DP of cotton cellulose measured by a viscometer after nitration was reported to be equal to 4700 [89]. However, it is well known that the DP of cotton is ~10,000 [2], suggesting that significant hydrolysis occurred to the cellulose chains in cotton during derivatization. The viscometric average

DP of aspen cellulose was determined to be 4581 after isolation via sodium chlorite pulping and NaOH hydrolysis [76]. This DP is comparable with that of aspen cellulose nitrate (DP = 5000); thus, the numbers in Table 9 appear to be reasonable DP values for native wood celluloses [89]. Furthermore, in cotton, a DP of 10,000 to 15,000 for secondary wall cellulose and DP of 2500 to 4000 and 250 to 500 for primary cell wall cellulose were reported [92, 93]. However, primary cell walls contain lower cellulose (20-25%) than secondary cell walls, which contain >95% cellulose [94].

Cellulose tricarbanilate (CTC) is another cellulose derivative for DP measurement, which is usually done by GPC [78, 82, 95]. CTC is the most utilized derivative for GPC studies due to following advantages: 1) complete substitution, 2) no depolymerization occurs during derivatization, 3) stability of the derivative, and 4) solubility and stability in THF [78]. Cellulose tricarbanilation is commonly performed by reaction of cellulose with phenyl isocyanate in either dimethylsulfoxide (DMSO) or pyridine as the solvents. However, it has been shown that cellulose oxidation and degradation occur during derivatization of cellulose in the presence of DMSO but not in pyridine [96]. Therefore, pyridine is most commonly used solvent for the derivatization, since it is important not to detrimentally affect its chain length during the isolation and preparation of samples. Typically, dried cellulose sample is derivatized by adding anhydrous pyridine and phenyl isocyanate, and kept at 65 °C with stirring until the cellulose is completely dissolved. Afterwards, methanol is added to the reaction mixture to eliminate the unreacted phenyl isocyanate. The mixture is then poured into a 3:7 water-methanol mixture to precipitate the cellulose tricarbanilate. The derivatized cellulose is finally purified by repeated washing with water-methanol followed by water [82].

The viscometry technique involves dissolving cellulose in 0.5 M cupriethylenediamine solution. According to the TAPPI T 230 om-08 method, dry cellulose is added to distilled water and followed by cupriethylenediamine solution, with continuous nitrogen flushing. Shaking and heating might be required to solubilize cellulose. The intrinsic viscosity is then measured for the resulting homogeneous solution.

DP values of several native woody and non-woody cellulose samples are listed in Table 10 [70, 74-76, 83]. It appears from the data in Table 10 that viscometry is more popular in determining DP of cellulose in lignocellulosic biomass. In fact, Kumar *et al*., stated that the viscometry technique is more adequate to analyze cellulose DP, given the complexity of lignocellulosic biomass [70]. The cellulose DP range is ~1500 – 4500. Hardwoods, such as poplar and aspen, have a cellulose DP of 3500 and 4500, respectively. Sweet *et al.* reported a DP of 1450 for southern pine, which is significantly less than the DP values reported for hardwoods [75]. Agricultural residues varied over a range of 1800 – 4000. Cellulose in jute fiber has a large DP value when compared to corn kernels, cotton stalks, wheat straw, and rice straw. DP of Nalita cellulose increased from ~3200 to 3600 as the tree became older (12 months to 30 months), suggesting that cellulose DP increases as the tree develops [76]. Nalita is a common name to *Trema orientalis*, a fast-growing shade tree with soft foliage, found in warm and wet regions of southern Africa and Asia [97].

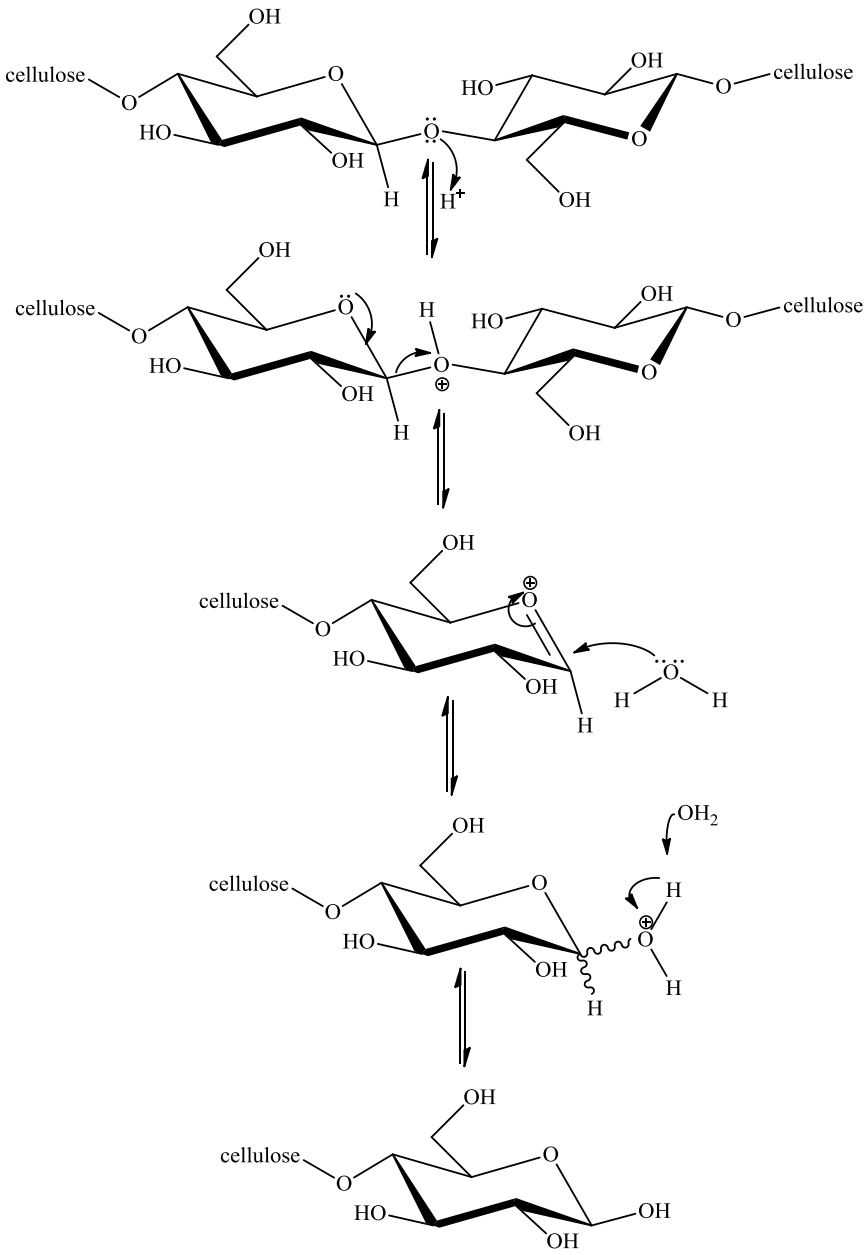
Table 10. DP of native wood and non-woody celluloses [70, 74-76, 83].

Species	Measurement Technique	DP
Southern Pine	CTC[a] and GPC	1450
DDGS	Cuen and Viscometry[b]	2243
Corn kernels	Cuen and Viscometry	1693
Dhaincha	Cuen and Viscometry	2520
Cotton stalks	Cuen and Viscometry	1820
Jute fiber	Cuen and Viscometry	3875
Wheat straw	Cuen and Viscometry	2660
Rice straw	Cuen and Viscometry	1820
Corn stover	Cuen and Viscometry	2520
Poplar	Cuen and Viscometry	3500
Aspen	Cuen and Viscometry	4581
Nalita (12 months)	Cuen and Viscometry	3181
Nalita (18 months)	Cuen and Viscometry	3383
Nalita (24 months)	Cuen and Viscometry	3518
Nalita (30 months)	Cuen and Viscometry	3611

[a]Cellulose tricarbanilate, [b]Viscometry technique gives DP_v

2.2.2.3.3 Level-Off Degree of Polymerization

When cellulose is subjected to acid hydrolysis, the degree of polymerization decreases rapidly until it reaches the so-called "leveling-off" or "limiting" degree of polymerization [98]. The acid-catalyzed hydrolysis of cellulose is depicted in Equation 3.



Equation 3. Acid-catalyzed hydrolysis of cellulose.

The DP of a cellulose sample is rapidly reduced to a relatively constant value upon being subjected to severe hydrolyzing treatments under conditions that do not produce humic substances [99]. The resulting cellulose fragments will have the maximum length that will allow them to dissolve in the particular hydrolyzing medium used [99]. The initial rapid DP degradation phase corresponds to the hydrolysis of the reactive

amorphous region of cellulose; while the slower plateau rate phase corresponds to the hydrolysis of the slowly reacting crystalline fraction of cellulose [98]. The acid hydrolysis conditions (acid concentration, temperature, and time) employed are dependent on the nature of the starting cellulose material [100]. Values between 1 – 4 M HCl, 60 – 80 °C, and 5 – 100 h are typically employed [100]. Table 11 summarizes typical LODP values associated with the cellulose materials as well as the hydrolysis conditions used [99].

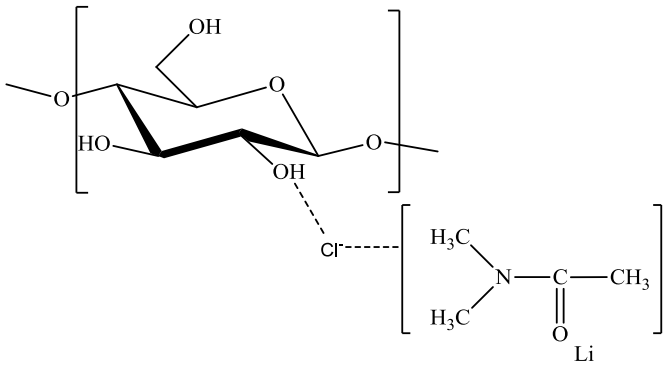
Table 11. Typical level-off degree of polymerization of some cellulose materials [99].

Form of Cellulose	LODP[a]
Ramie	350 – 300
Cotton	250 – 200
Unbleached sulfite wood pulps	400 – 250
Bleached sulfite pulps	280 – 200
Bleached sulfate wood pulps	190 – 140

[a]Based on hydrolysis in 2.50 *N* HCl at 105 °C for 15 minutes

2.2.2.3.4 Light Scattering Measurements of Cellulose DP

In order to obtain absolute molecular weight of cellulose and thus DP, light scattering techniques are usually applied. Size exclusion chromatography (SEC) coupled with a multi-angle laser light scattering (MALLS) is the system typically used for the light scattering measurement. The mobile phase and the solvent used to dissolve cellulose is either lithium chloride/*N*,*N*-dimethylacetamide (LiCl/DMAc) or lithium chloride/1,3-dimethyl-2-imidazolidinone (LiCl/DMI). The solubility of cellulose in LiCl/DMAc, for example, is due to the association of the hydroxyl protons of the anhydroglucose units with the chloride anion by hydrogen bonding, while the chloride ion is associated with a Li^+(DMAc) macrocation as follows [101]:



In such system, no cellulose derivatization is required. The former solvent system has few disadvantages that led to the development of the latter solvent system [102]. LiCl/DMAc causes: (1) aggregation of cellulose in the solution, which is dependent on the concentration of cellulose or LiCl [103, 104], (2) incomplete dissolution of certain cellulose samples, such as tunicate cellulose [105] and softwood bleached kraft pulps [106], (3) detrimental degradation of cellulose upon heating during dissolution in LiCl/DMAc [107]. On the other hand, LiCl/DMI has shown superior advantages over LiCl/DMAc, such as stability of cellulose and solubility of tunicate cellulose and kraft pulps [102, 108].

SEC-MALLS analysis has been mostly performed on cellulose-rich samples, because dissolving native wood is still difficult to achieve [108]. Table 12 summarizes some of the samples analyzed by SEC-MALLS with their respective DP values [108-110]. The samples were dissolved in 8% LiCl/DMI. As shown in Table 12, the samples include native cellulose, such as cotton lint cellulose, algal cellulose, and bacterial cellulose, and a variety of bleached and unbleached pulps (kraft or sulfite).

Table 12. DP of several cellulose-rich samples after dissolution in LiCl/DMI using the SEC-MALLS method [108-110].

Species	DP
Bleached SW kraft pulp	4500
Bleached HW kraft pulp	3000
Bleached spruce sulfite pulp	3000
Cotton lint cellulose	2600
Algal cellulose	4300
Bacterial cellulose	7300
Unbleached SW kraft pulp	3700
Oxygen-bleached SW kraft pulp	3140
Chlorine-bleached SW kraft pulp	2510
Alkali-extracted SW kraft pulp	3170
ClO_2-bleached SW kraft pulp	2300
Unbleached SW sulfite pulp	1640
Unbleached HW kraft pulp	1590
H-M cotton linter	64
H-Tencel[a]	44
H-Bemliese[a]	35
H-M-Tencel[a]	46
H-M-Bemliese[a]	41

[a]Tencel and Bemliese were mercerized (M) (alkali-treated) with 20% NaOH, and acid hydrolyzed in 1 M HCl at 105 °C for 3 h (i.e. H-M-Tencel and H-M-Bemliese).

Tencel is regenerated cellulose fiber prepared from dissolving wood pulp using the *N*-methylmorpholine-*N*-oxide system, while Bemliese is regenerated cellulose fiber prepared from cotton linters using the cuprammonium solvent system [110]. Tencel and Bemliese were mercerized (M) (alkali-treated) with 20% NaOH, and acid hydrolyzed in 1 M HCl at 105 °C for 3 h (i.e. H-M-Tencel and H-M-Bemliese) [110].

2.2.3 Pretreatment

Since biomass is naturally resistant to biological degradation, pretreatment is required to alter the structure of cellulosic biomass to make cellulose more accessible to enzymes that convert the carbohydrate polymers into fermentable sugars [111]. Enzymatic conversion of cellulose to glucose is a very slow process because the native cellulose is well protected by a matrix of hemicellulose and lignin (Figure 17); therefore, it is difficult for the enzymes to attack the cellulose polymer and break it down to the monomer sugars [7].

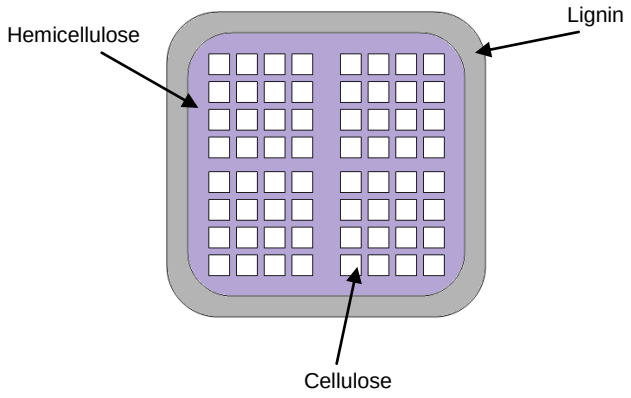


Figure 17. Cellulose morphology.

In order to make the enzyme-catalyzed conversion of cellulose to glucose more efficient, the cellulose must be more accessible and reactive to the enzyme (cellulases), and this is done by the pretreatment step [7, 111]. In general, pretreatment should be capable of hydrolyzing/de-acetylating hemicelluloses, hydrolyzing lignin, reducing the crystallinity and DP of cellulose, increasing the porosity and surface area of the lignocellulosic material [8, 20]. Therefore, the pretreatment of the raw material is probably the most important step as it has a large impact on all the other steps in the

process of producing ethanol from biomass [7]. Figure 18 illustrates the effects of pretreatment on lignocellulosic materials [111].

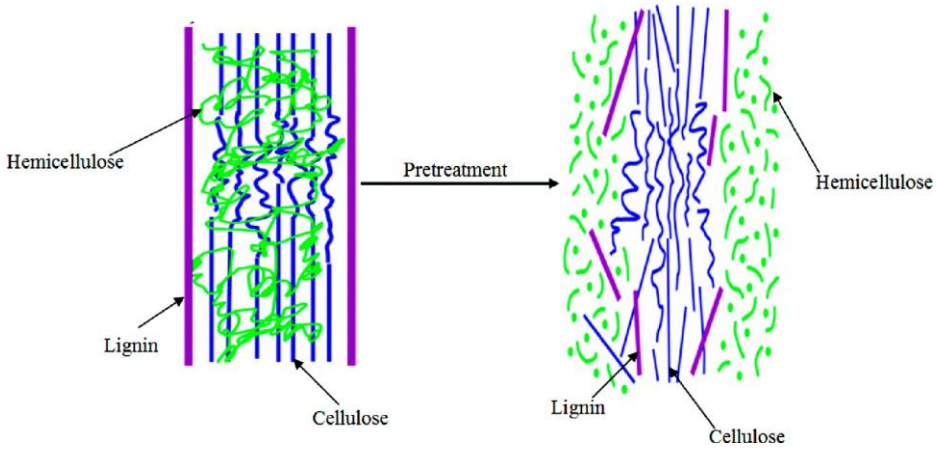


Figure 18. Schematic of goals of pretreatment on lignocellulosic material [111].

Ideally, an effective pretreatment method has to [7]:

- ✓ Result in high recovery of all carbohydrates
- ✓ Result in high digestibility of the cellulose in the subsequent enzymatic hydrolysis
- ✓ Produce no or very limited amounts of sugar and lignin degradation products. The pretreatment liquid should be possible to ferment without detoxification
- ✓ Have a low energy demand
- ✓ Have a low operational/capital cost

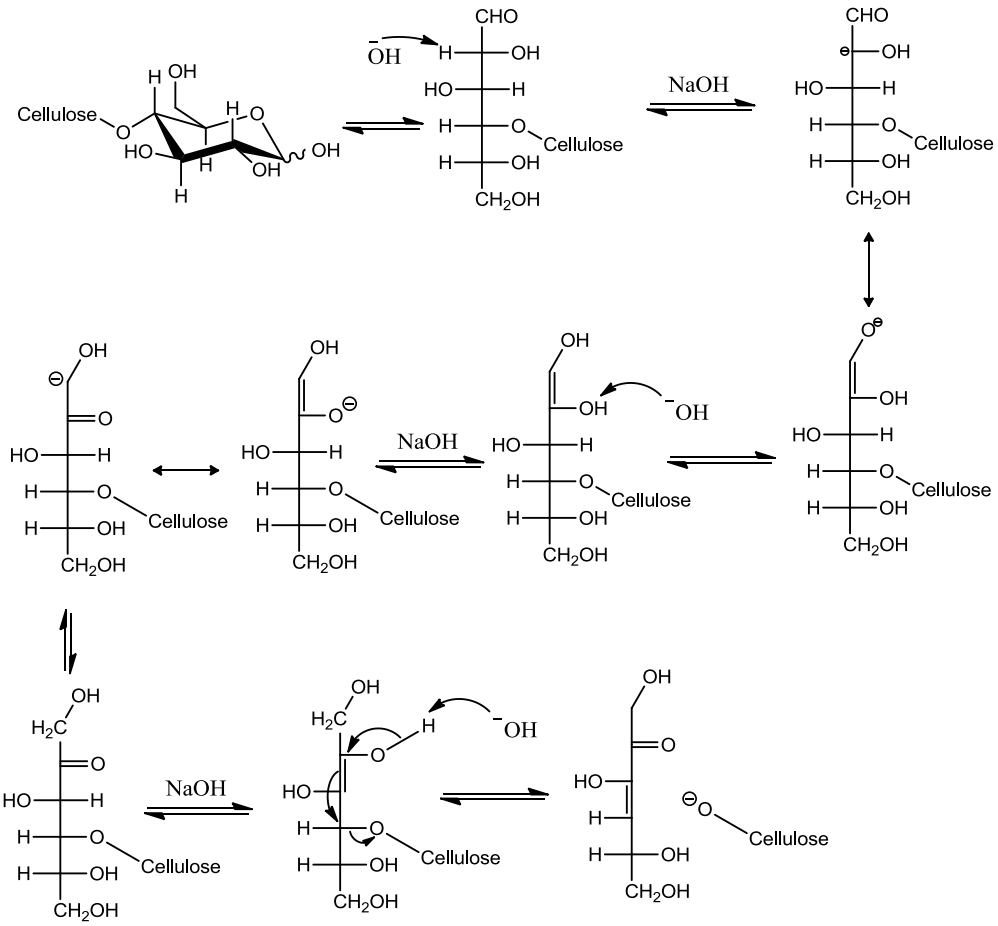
2.2.3.1 Pretreatment Methods

This section will discuss several pretreatment technologies: their operational conditions, fundamental chemistry, and effects on lignocellulosic materials.

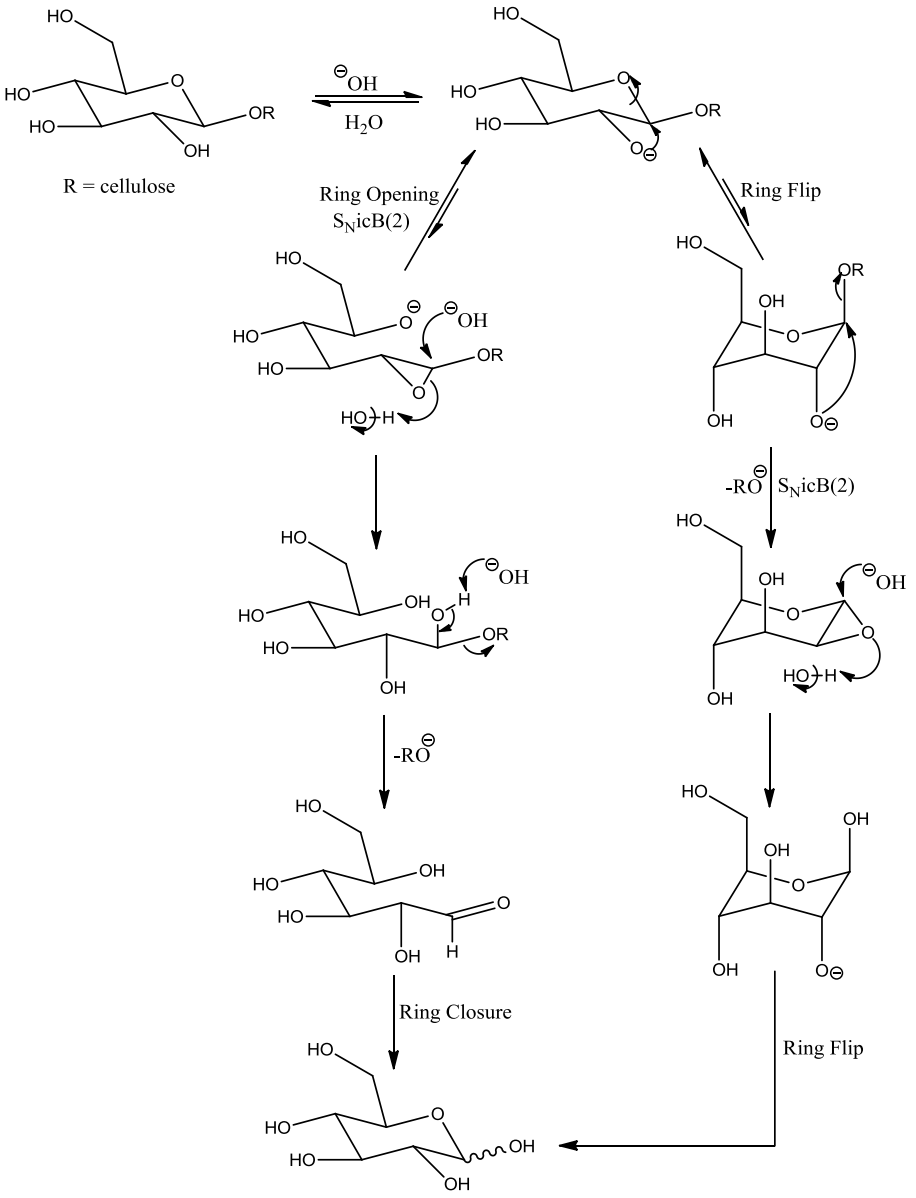
2.2.3.1.1 Alkali Pretreatment

Alkali pretreatment is one of the chemical methods that has been widely used to prepare biomass for enzymatic hydrolysis. Many alkali reagents have been used, including sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide (lime) ($Ca(OH)_2$), ammonia (NH_3), and urea ($(NH_2)_2CO$) [112, 113]. Sodium hydroxide has received most attention because, as a strong base, it effectively enhances lignocellulosic digestibility [111, 112]. When corn stover was pretreated with aqueous NaOH, high sugar yield (63.7 g per 100 g corn stover) were obtained from the enzymatic hydrolysis of the pretreated substrate [114]. The high sugar yield was due to the fact that 95% of the lignin and 88% of the hemicellulose were removed from the solid material after the biomass was pretreated with 10 wt % NaOH at 120 °C for 60 min [114].

When the biomass is pretreated with an alkaline solution, such as NaOH, the internal surface area of cellulose increases, and the degree of polymerization and crystallinity decrease [7, 20]. Alkali pretreatment causes peeling reaction and chain cleavage reactions, which lower the degree of polymerization and crystallinity of cellulose. The pealing reaction is the loss of D-glucose units by successively removing reducing end sugar units, and the chain cleavage reaction is cleaving the β-1, 4-glycosidic bonds. Equation 4 explains the peeling reaction and Equation 5 illustrates the mechanism for the base-catalyzed cleavage of cellulose.



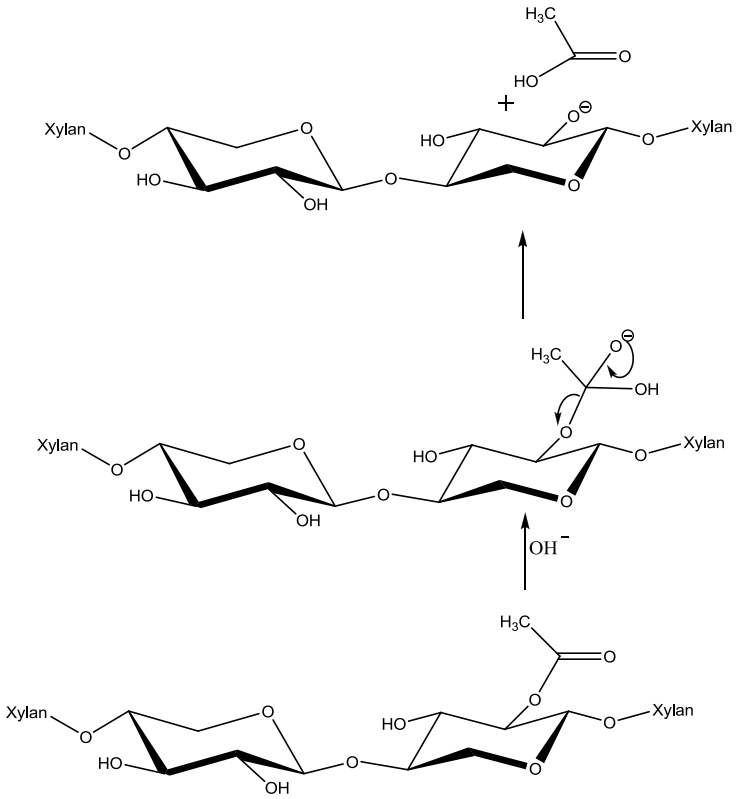
Equation 4. Mechanism for the peeling reaction of cellulose under alkali condition.



Equation 5. Mechanism for the base-catalyzed cleavage of cellulose. The $S_N$icB(2) refers to a nucleophilic substitution by an internal nucleohpile – the conjugate base of the C2 hydroxyl group.

In addition, the alkaline solution fragments and solubilizes lignin and the hemicellulose; the hemicellulose is usually recovered as oligomers [7]. After the alkali pretreatment, the carbohydrates become more accessible to enzymatic attack as the alkalis break the bonds between the lignin and the carbohydrates, and remove the acetate

groups and uronic acids from the hemicellulose (hardwood xylan), which cause steric hindrance [7, 111, 112]. Equation 6 shows the removal of the acetate groups from hardwood xylan.



Equation 6. Base-catalyzed removal of acetate groups from xylan.

Alkali pretreatment works mainly by delignification; therefore, it is more effective when used on agricultural residues (corn stover) and hardwood species (poplar) than softwood species (pine) because the amount of lignin in softwood species is higher [7]. Alkaline pretreatment on switchgrass [112], wheat straw [113], poplar [115], and corn stover [116] were reported. As mentioned earlier many alkalis have been used for the pretreatment of biomass. However, from an industrial and economical point of view, calcium hydroxide (lime) has been reported to be more advantageous than the other

reagents. Table 13 compares four alkalis based on cost, handling, efficiency in enhancing biomass digestibility, and recovery [112, 113].

Table 13. Comparison between four alkali reagents based on cost, handling, efficiency in enhancing biomass digestibility, and recovery [112, 113].

	Cost	Handling	Efficiency in enhancing biomass digestibility	Recovery
NaOH	Expensive ($0.68/Kg)	Dangerous	Significant	Difficult
$Ca(OH)_2$	Inexpensive ($0.06/Kg)	Safe	Significant under certain pretreatment conditions	Easy
NH_3	Moderately expensive ($0.25/Kg)	Safe – careful handling	Low	Easy
$(NH_2)_2CO$	Modestly expensive ($0.17/Kg)	Safe	Low	----

Lime or calcium hydroxide ($Ca(OH)_2$) is a preferred reagent because it is relatively inexpensive, safe to handle, and easy to recycle. Chang *et al*. developed a method to easily recover lime [113]. After the pretreatment, the biomass is washed with water, and CO_2 is then added to the water to form insoluble $CaCO_3$, which can be separated from liquid and converted to lime using lime kiln [113]. Based on the significant advantages that lime provides, it has been used for the pretreatment of bagasse, wheat straw, switchgrass, and corn stover, since these herbaceous crops have low lignin content [112, 113, 116]. The lime pretreatments of bagasse, wheat straw, switchgrass, and corn stover were studied. Researchers tried to optimize the process by monitoring the effects of pretreatment conditions on digestibility. As the process variables (time, temperature, lime loading, water loading, and biomass particle size) were systematically varied, the amount sugar yields were calculated. Table 14 summarizes the

recommended conditions that were concluded from the lime pretreatment of bagasse, wheat straw, switchgrass, and corn stover [112, 113, 116].

Table 14. Recommended lime pretreatment conditions for baggase, wheat straw, switchgrass, and corn stover [112, 113, 116].

	Time (hours)	Temperature (°C)	Lime loading (g $Ca(OH)_2$/g dry biomass)	Water loading (mL/g dry biomass)	Biomass particle size (mm)
Bagasse	1	120	0.1	10	< 0.420
Wheat straw	1	125	0.1	10	< 0.420
Switchgrass	2	120	0.1	9	< 0.420
Corn stover	2	140	0.1	10	< 0.420

Enzymatic hydrolysis was performed on the lime pretreated biomass and a comparison between the amount of sugar yields before and after the lime treatment are summarized in Figure 19 [112, 113, 116].

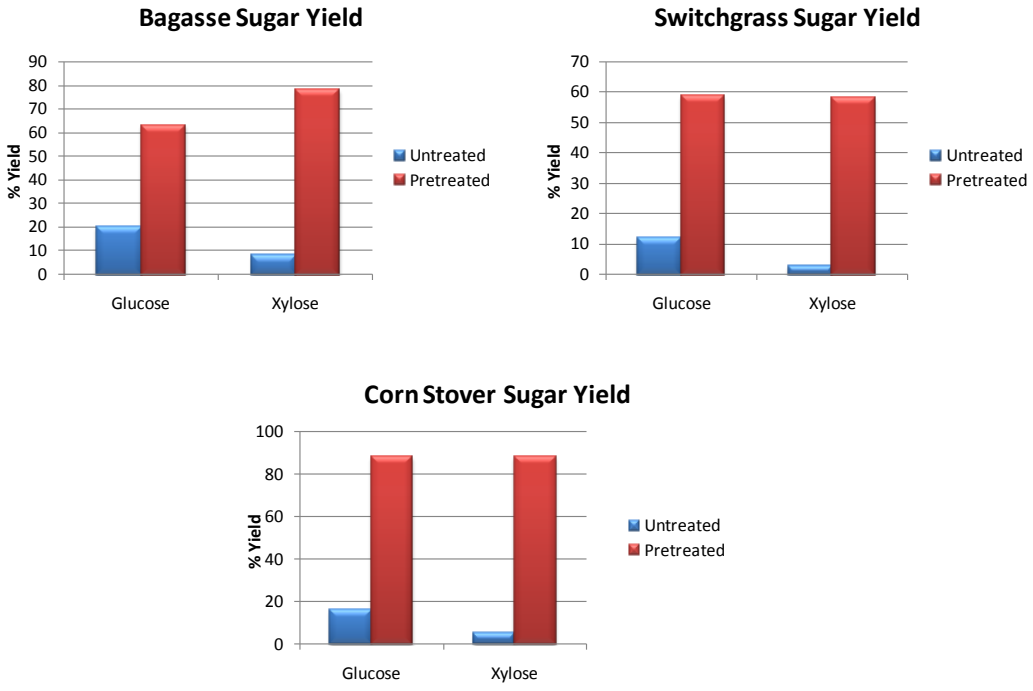


Figure 19. Sugar yields from lime pretreated bagasse, switchgrass, and corn stover after enzymatic hydrolysis [112, 113, 116].

The results in Figure 19 clearly represent the goals of pretreatment; there is a significant increase in the sugar yields when the biomass was pretreated with lime. Not only does lime pretreatment increase sugar yields, but it is also mild (Figure 20) [112, 113, 116].

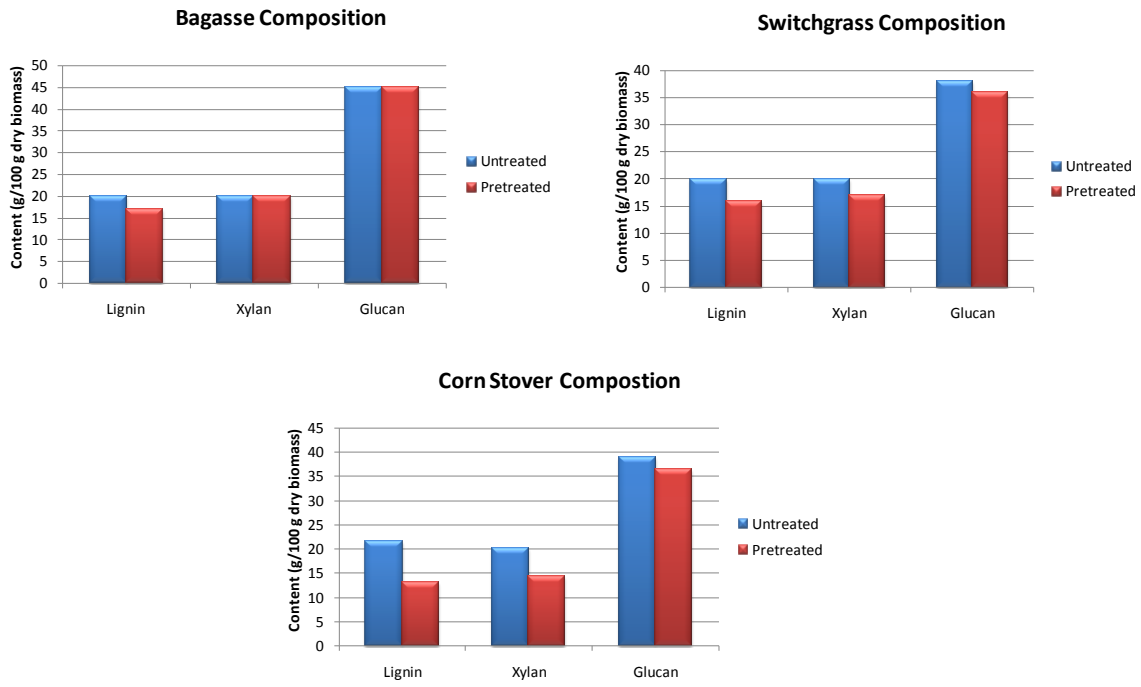


Figure 20. Composition of untreated and lime pretreated baggasse, switchgrass, and corn stover [112, 113, 116].

Lime pretreatment causes no or little removal of xylan and glucan, but it removes about 18% of lignin. This again shows that lime, or alkali pretreatment in general, acts mainly by delignification, which makes it unsuitable for the pretreatment of biomass with high lignin content such as softwoods. For hardwood, such as poplar, lime pretreatment can be used but requires the addition of oxygen or air [117]. This combination solubilizes significant portions of the lignin (~80%), which enhances the biomass digestibility [117, 118]. The recommended process conditions are: oxygen pressure = 14.0 bar, time = 6 h,

temperature 150 °C, particle size < 0.420 mm, 0.1 g of $Ca(OH)_2$/g of dry biomass, and 9 mL of water/g of dry biomass [115]. The sugar yields from the enzymatic hydrolysis of pretreated poplar were greater than the yields from the untreated substrate (Figure 21) [115].

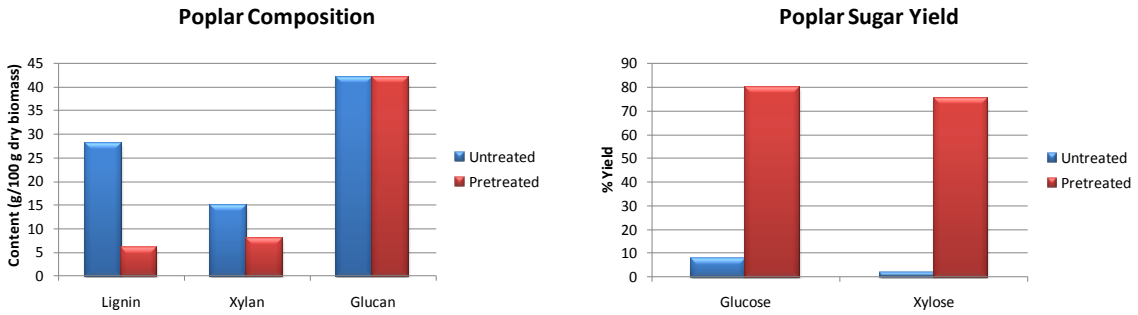


Figure 21. Composition of untreated and lime pretreated poplar; and the sugar yields after enzymatic hydrolysis [115].

The major effect of the alkali pretreatment is the removal of lignin from the biomass, thus improving the biomass digestibility [111]. Therefore, alkali pretreatment is only suitable for biomass with low lignin content such as corn stover and poplar. Alkali pretreatments remove acetate and uronic acid groups from the hemicellulose, which allows the surfaces of cellulose and hemicellulose be more accessible to the enzymes [111]. Alkali pretreatment requires low temperatures because at high temperatures carbohydrate degradation can occur, which causes the formation of organic acids, thus neutralizing the alkalis [115]. Alkali pretreatments have not been commercialized due to the cost and difficulty to recover the base. A significant disadvantage of alkaline pretreatment is the conversion of alkali into irrecoverable salts and/or the incorporation of salts into the biomass during the pretreatment reactions so that the treatment of a large amount of salts becomes a challenging issue for alkaline pretreatment [119].

2.2.3.1.2 Dilute Acid Pretreatment

Typical conditions for dilute sulfuric acid pretreatment are in the following ranges: 0.1 – 2.0% w/w acid, 120 – 230°C, and 1 – 40 min [4, 20, 120, 121]. The main effect of acid pretreatment is hemicelluloses hydrolysis, which in turn makes cellulose more accessible to enzymatic digestibility [111, 117]. Figure 22 shows the acid-catalyzed hydrolysis of xylan. Pretreatment severity is a single mathematical value that combines the various pretreatment conditions of time, temperature and/or acid concentration. It is typically defined as the combined severity factor (log CS) according to the following equations:

$$R_0 = t.\exp[(T_H - T_R)/14.75]$$

$$\log CS = \log R_0 - pH$$

where R_0 is the severity factor, t is the reaction time in minutes, T_H is the hydrolysis temperature in °C, and T_R is a reference temperature, most often 100 °C [122]. Xylan removal/hydrolysis increases as the pretreatment severity increases [123]. For example, xylan removal increased from 8% to >90% when corn stover was pretreated under condition with severity values ranging from 2.5 to 4.5 [123]. The pretreatment was carried in a batch reactor with reaction temperatures of 160°C, 180°C, 200°C, and 220°C; 0.0 wt% and 0.1 wt% H_2SO_4, with the solids concentration held at 5 wt% [123]. The effect of xylan removal on the enzymatic hydrolysis of corn stover is illustrated in Figure 23, which clearly shows a direct relationship; the enzymatic digestibility increased with more removal/hydrolysis of xylan [123].

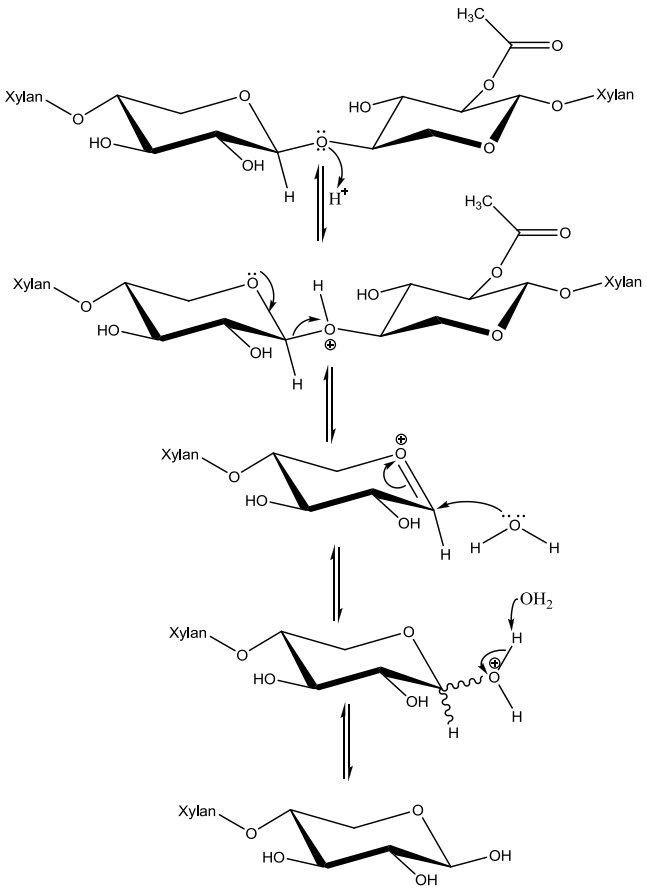


Figure 22. Acid-catalyzed hydrolysis of xylan.

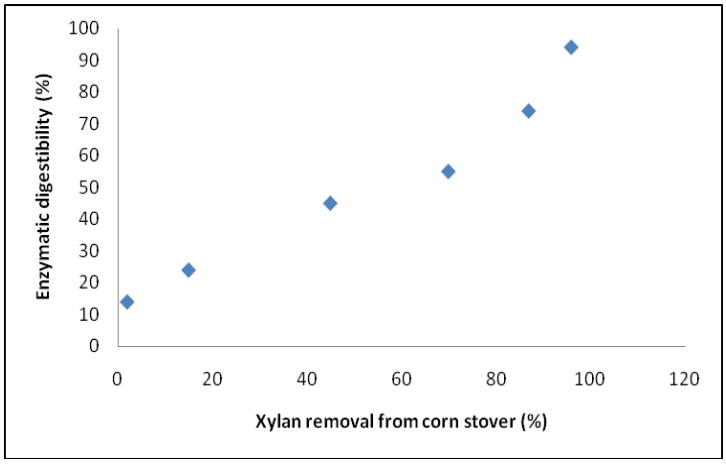


Figure 23. Effect of xylan removal on the enzymatic digestibility of corn stover after dilute acid pretreatment [123].

Achieving high xylan to xylose conversion yields during dilute acid pretreatment is necessary to achieve favorable overall process economics because xylan accounts for up to one-third of the total carbohydrate in many lignocellulosic materials [124]. Thus, finding optimum dilute acid pretreatment conditions is typically based on maximum xylose yield/recovery, followed by efficient enzymatic hydrolysis of the pretreated solids. Table 15 shows the xylose recovery and enzymatic hydrolysis after the optimum pretreatment conditions for corn stover, switchgrass, and poplar [125, 126].

Table 15. Optimum dilute sulfuric acid pretreatment conditions for several biomass, sugar yields, and the efficiency of enzymatic hydrolysis.

Biomass	Pretreatment conditions			Sugars yield (%)		Enzymatic conversion (%)	
	Acid (%)	Time (min)	Temperature (°C)	Xylose	Glucose	Untreated	Pretreated
Corn stover [125]	2.0	43	120	77	8.4	-	70[a]
Switchgrass [126]	1.2	0.5	180	80.8	17.9	18[b]	91.4[b]
Poplar [126]	1.0	0.56	180	81.9	5.8	10[b]	79.1[b]

[a]Enzymatic hydrolysis conditions: 22 FPU/g substrate, temp. of 49 °C, pH 4.8, and a reaction time of 38 h.
[b]Enzymatic hydrolysis condition: 60 FPU/g cellulose, temp. of 50 °C, pH 5.0, and a reaction time of 8 days.

The compositions of the pretreated switchgrass and poplar solids are summarized in Table 16. It clearly shows that dilute sulfuric acid pretreatment acts mainly by the removal of hemicellulose and that the lignin content in the biomass stays relatively high. However, it has been shown that the lignin structure is modified during pretreatment. Such modifications include increase in degree of condensation and cleavage of lignin interlinkages [11, 65].

Enzymes, such as cellulase, adsorb non-productively on lignin, thus lowering the productive binding with cellulose. If bovine serum albumin (BSA) is added to the

biomass after the acid pretreatment, BSA will adsorb competitively and irreversibly on lignin and the effectiveness of cellulase will improve [127]. A study showed that the addition of BSA prior to enzymatic hydrolysis improved enzymatic conversion of cellulose to glucose [127]. Corn stover was pretreated with sulfuric acid (1 wt%) at 140 °C for 40 min. With the addition of BSA (1% w/w), the cellulose conversion improved from 82% to 92% at a cellulase loading of 15 FPU/g cellulose [127].

Table 16. Glucan, xylans, and klason lignin content in untreated and dilute H_2SO_4 pretreated corn stover, switchgrass, and poplar [126, 127].

	Feedstock composition (%, dry wt. basis)			Composition of pretreated solids (%, dry wt. basis)		
	Corn stover	Switchgrass	Poplar	Corn stover[a]	Switchgrass[b]	Poplar[c]
Glucan	37.8	32.2	39.8	56	49.7	54.9
Xylan	21.3	20.3	14.8	11.2	2.2	1.5
Klason lignin	17.8	19.5	26.9	28.2	34.9	35.9

[a]Pretreatment conditions: 1% H_2SO_4 (%w/w), 40 min, and 140°C.
[b]Pretreatment conditions: 1.2% H_2SO_4 (%w/w), 0.5 min, and 180°C.
[c]Pretreatment conditions: 1.0% H_2SO_4 (%w/w), 0.56 min, and 180°C.

A study by Selig *et al*. showed that during dilute acid pretreatment of corn stover, lignin droplets are produced, which may retard enzymatic hydrolysis of cellulose [128]. On the other hand, the formation of lignin droplets could mean removal of lignin and could increase accessible cellulose. At high-temperature pretreatment (120 – 200 °C), lignin migrates from the cell wall to the bulk liquid phase followed by deposition onto the biomass surface [128]. At temperatures above the phase transition temperature for lignin (120 – 200 °C), lignin is fluidized and mobility permitted within the confines of the cell wall matrix [128]. Once in the liquid phase, cell wall lignin may be forced to coalesce in an aqueous environment, and then could migrate from the cell wall to the bulk liquid phase of the pretreatment mixture due to capillary and hydrophobic forces [128]. When

the mixture is cooled, coalesced lignins could harden and either become trapped within the cell wall layers or settle out of the bulk liquid phase, potentially depositing back onto the biomass surface [128].

2.2.3.1.3 Organosolv Pretreatment

In an organosolv pretreatment, the internal bonds between lignin and hemicellulose are broken, hemicelluloses and lignin are hydrolyzed by treating the biomass in an organic or aqueous-organic solvent mixture with the addition of an inorganic acid catalyst, such as H_2SO_4 or HCl [7]. Typically, solvents such as methanol, ethanol, acetone, ethylene glycol, triethylene glycol, and phenol are used in the organosolv process [7]. After pretreatment, the hydrolyzed lignin dissolves in the organophilic phase and recovered as the filtrate, the cellulose is recovered as the solid residue, and the hemicellulose is recovered in the water-soluble fraction as monomeric and oligomeric sugars [7, 129].

Organosolv pretreatment is of interest because it has several advantages: (1) organic solvents are easy to recover by distillation and recycled for pretreatment; (2) the recovered lignin after pretreatment has desirable characteristics that can be used for several coproducts; (3) the pretreatment considers the utilization of all the biomass components, which makes it feasible for biorefining of lignocellulosic biomass [130, 131].

Organosolv pretreatment of hardwood [129] and softwood [132] were investigated. Pan and co-workers have extensively studied the ethanol organosolv pretreatment of poplar and pine, examined the effects of various process parameters on the yield and distribution of cellulose, hemicellulose, and lignin in the three fractions

(solid fraction, ethanol organosolv lignin fraction, and water-soluble fraction) [129, 132]. The process parameters were cooking temperature (T), time at the cooking temperature (t), sulfuric acid concentration (S), and ethanol concentration (C). Typical conditions are within the following ranges: T = 150 – 200°C; t = 30 – 90 min; S = 0.5 – 1.5% w/w; C = 25 – 80% v/v [129, 132, 133].

Finding the optimum conditions was based on recovering solids with high glucose content and low lignin content, high recovery of the hemicelluloses in the water fraction, as well as efficient enzymatic hydrolysis of the solid substrate. Table 17 summarizes the composition of the three fractions after ethanol organosolv pretreatment of poplar and pine at the optimum conditions, and compared these values to the untreated raw materials. In both cases, the resulting solid substrates of poplar and pine mainly contain glucose with minimal amounts of lignin and other sugars such as xylose and mannose. Most of the hemicelluloses were recovered in the water soluble fraction, which also contains considerable amounts of soluble lignin due to lignin hydrolysis. However, the majority of the lignin is recovered in the EOL fraction. The resulting solid substrate is highly reactive toward enzymatic hydrolysis partially because most of the hemicelluloses and lignin are removed and the remaining cellulose is very accessible to enzymatic digestibility. At enzymes loading of 20 FPU cellulose/g cellulose and 40 IU β-glucosidase/g cellulose, the conversion of cellulose to glucose in ethanol organosolv pretreated poplar and pine to glucose was 98% [129, 132].

Table 17. Compositional analysis of the raw materials and the three fractions after ethanol organosolv pretreatment of poplar and pine at the optimum conditions [129, 132].

Content[a]	Untreated poplar	Pretreated poplar[b]	Untreated pine	Pretreated pine[c]
EOL		**15.53**		**19.57**
Solid		**54.19**		**43.05**
Klason lignin	**20.95**	5.88	**24.79**	4.17
Acid-soluble lignin	**2.30**	0.30	**0.29**	0.1
Glucose	**48.95**	43.16	**50.46**	37.63
Xylose	**17.85**	3.36	**7.21**	0.56
Mannose	**3.388**	1.49	**13.09**	0.59
Galactose	**0.38**	-	**2.22**	-
Arabinose	**0.26**	-	**1.42**	-
Water-soluble		**16.98**		**20.06**
Acid-soluble lignin		5.22		4.76
Glucose		0.55		4.19
Xylose		9.37		3.23
Mannose		1.29		5.37
Galactose		0.33		1.73
Arabinose		0.22		0.78

[a] % (w/w) in oven-dried wood.
[b] Pretreatment conditions: 1.25 % w/w H_2SO_4, 50 % v/v ethanol, 180 °C, and 60 min.
[c] Pretreatment conditions: 1.10 % w/w H_2SO_4, 65 % v/v ethanol, 170 °C, and 60 min.

Organosolv lignins are more environmentally advantageous because they contain insignificant levels of sulfur and sodium, indicating very low ash content [130]. Both hardwood and softwood organosolv lignins have low molecular weights and narrow polydispersity; low polydispersity is important in controlling variability in co-product applications [130]. Also, softwood and hardwood organosolv lignins have a high level of phenolic hydroxyl groups, relative to lignins prepared using other processes [130]. However, softwood lignins have higher levels of phenolic groups than hardwood, thus making softwood lignins suitable for the production of phenolic, epoxy, and isocyanate resins [130]. Table 18 summarizes the characteristics of pine and poplar EOL.

Table 18. Weight-average molecular weight ($\overline{M}_n$), the number-average molecular weight ($\overline{M}_n$), polydispersity index (D) ($\overline{M}_w / \overline{M}_n$), and functional groups of pine and poplar EOL [134, 135].

	Molecular weight and polydispersity index[b]			Functional group[c], mmol/g lignin	
EOL[a]	$\overline{M}_n$	$\overline{M}_n$	D	ArOH	AlkOH
Pine	1280	3010	2.35	3.41	4.43
Poplar	1093	2105	1.93	3.48	3.85

[a] Ethanol organosolv lignin produced under 1.10 % w/w H_2SO_4, 65 % v/v ethanol, 170 °C, and 60 min for pine, and 1.25 % w/w H_2SO_4, 50 % v/v ethanol, 180 °C, and 60 min for poplar.

[b] Weight-average molecular weight ($\overline{M}_n$), the number-average molecular weight ($\overline{M}_n$), and the polydispersity index (D) ($\overline{M}_w / \overline{M}_n$)

[c] ArOH = phenolic hydroxyl group; AlkOH = aliphatic hydroxyl group.

2.2.3.2 Inhibition

Inhibitors are substances that bind to enzymes and decrease their activity. During the pretreatment of biomass, some substances are formed that inhibit cellulase activity, thus lowering the hydrolysis efficiency as well as the efficiency of sugar fermentation to ethanol. These compounds are formed due to the degradation of hemicellulose, cellulose, and lignin during the pretreatment. The amount of inhibitors formed depends on the severity of the pretreatment conditions, which include temperature, time, pressure, and reagent dosage. The main degradation pathways are schematically presented in Figure 24 [136].

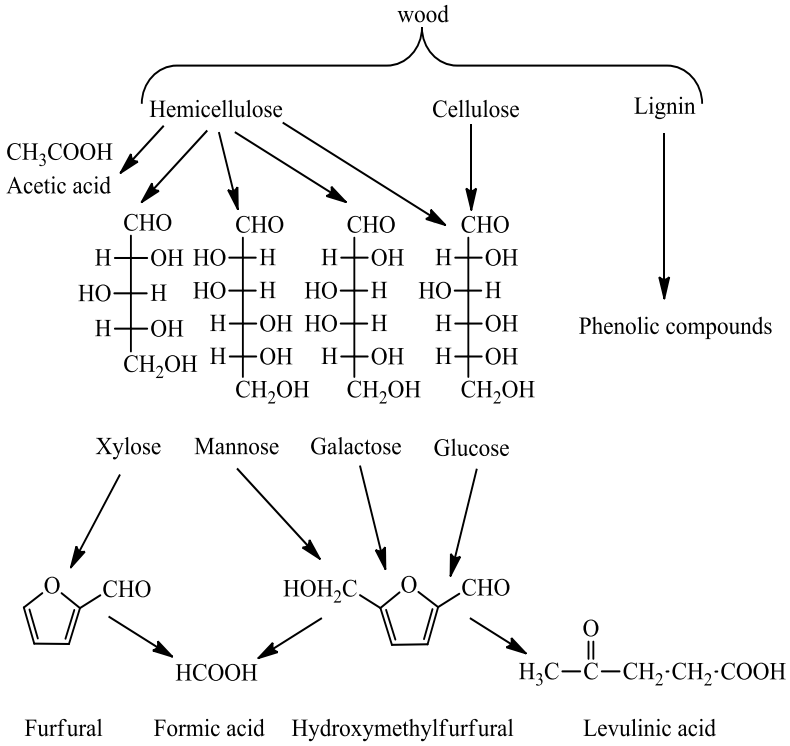


Figure 24. Reactions occurring during hydrolysis of lignocellulosic materials [136].

The inhibitors are usually formed during the pretreatments which employ high temperatures and acidic conditions, such as dilute sulfuric acid and organosolv pretreatments. During pretreatment, lignin is solubilized and degraded into phenolic compounds. Hemicellulose forms acetic acid, or the derived sugars can degrade to form various substances such as, furfural, 5-hydroxylmethylfurfural (5-HMF), formic acid, or levulinic acid. The reactions are depicted in Figure 25 [137].

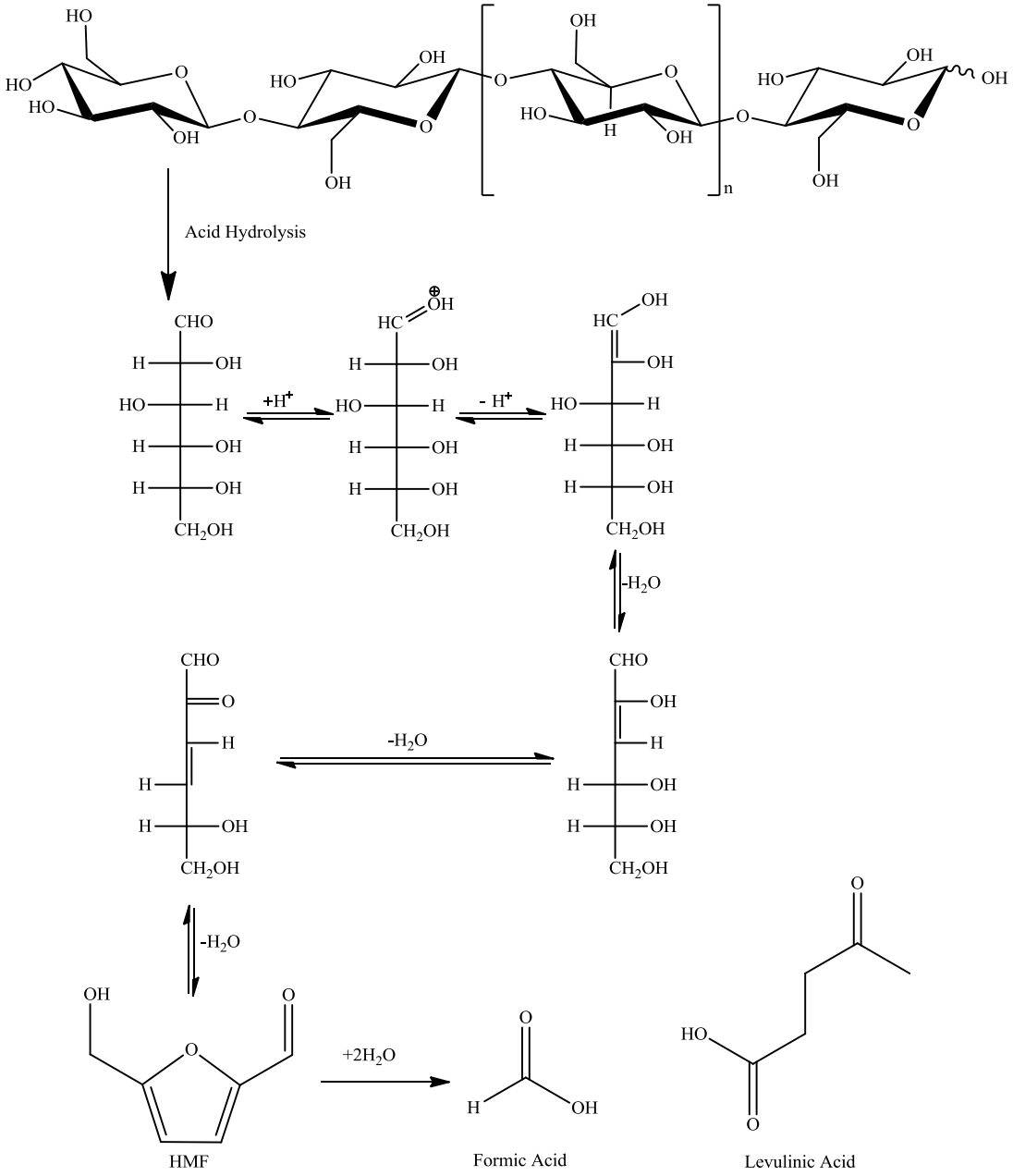


Figure 25. Schematic representation for the formation of HMF, formic and levulinic acids from cellulose during pretreatment [137].

As for alkali pretreatment, no inhibitors are produced, and that is one advantage of such pretreatment technologies. During organosolv pretreatment, the solvents must be removed from the system because they may be inhibitory to the enzymatic hydrolysis and fermentation [138].

Inhibitors can be controlled by adjusting the pretreatment conditions in a way that the formation of these inhibitors can be reduced. Therefore, the optimum conditions are those that produce high carbohydrate recovery and at the same time controlling the formation of inhibitors. Typically, the content of these inhibitors increases with increasing pretreatment severity. Figure 26 shows that the furans content increased with increasing the severity of the ethanol organosolv pretreatment of *Miscanthus* [133].

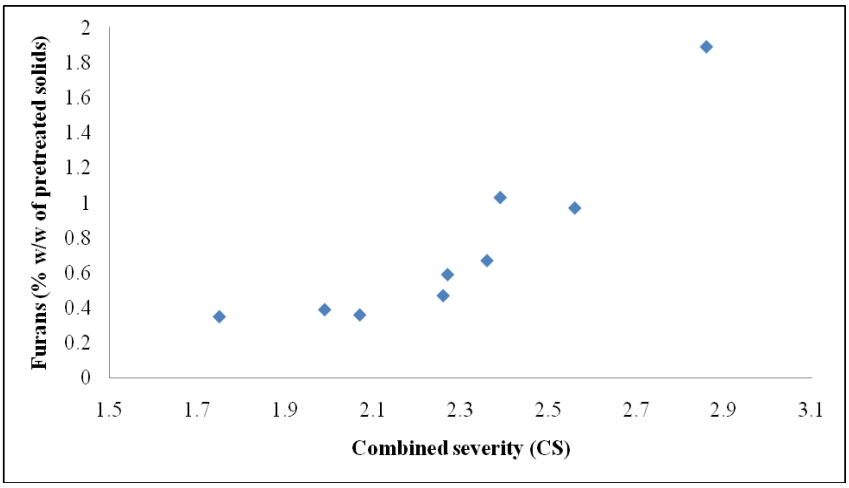


Figure 26. The effect of the ethanol organosolv pretreatment severity on the production of furans from *Miscanthus x giganteus* [133].

Detoxification is another way to control the production of inhibitors. Detoxification is basically the removal of inhibitors prior to fermentation [139]. Biological detoxification method uses enzymes such as, peroxidase and laccase to remove phenolic monomers and phenolic acids [140]. Physical detoxification method is described as the removal of inhibitors by extraction with diethyl ether or ethyl acetate [139]. However, this method is not commercially viable due to safety issues and cost to handle such chemicals. In a chemical detoxification method, the hydrolysates are treated with an alkali ($Ca(OH)_2$). In this process, the pH is increased to 9-10 by the addition of

$Ca(OH)_2$ (overliming) and then readjust the pH to 5.5 in order to perform the fermentation [139]. Overliming precipitates the toxic compounds with $CaCO_3$ and removes the inhibitors as they become unstable under high pH [139].

2.2.3.3 Effect of Pretreatment on the Cellulose Degree of Polymerization

In the realm of cellulosic ethanol, studies have been reported to examine the effect of pretreatment on DP of cellulose, which in turn affects the efficiency of the downstream enzymatic deconstruction step. Table 19 summarizes the DP of cellulose of various biomass after some pretreatment processes, such as ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute sulfuric acid, lime, SO_2, ozone (O_3), carbon dioxide explosion (CE), alkaline explosion (AE), and organosolv.

Table 19. DP of several biomass cellulose after various pretreatment technologies.

Biomass	Pretreatment	DP[a]
Corn Stover [70]	Ammonia fiber expansion	6800
	Ammonia recycled percolation	4500
	Controlled pH	5700
	Dilute Acid	2700
	Lime	3100
	SO_2	3000
Poplar [70]	Ammonia fiber expansion	2600
	Ammonia recycled percolation	3100
	Controlled pH	1800
	Lime	1500
	SO_2	500
Bagasse [91]	O_3	800
	CO_2 explosion	572
	Alkaline explosion	550
Wheat Straw [91, 141]	O_3	908
	CO_2 explosion	698
	Alkaline explosion	662
	Organosolv (acetic acid)	1594
	Organosolv (formic and acetic acid)	2182
	Organosolv (methanol)	1519
	Organosolv (ethanol)	1356
Eucalyptus regnans [91]	O_3	1065
	CO_2 explosion	815
Pinus radiata [91]	O_3	2900
Spruce:Pine (50:50) [73]	Dilute acid	200

[a] DP was measured using either nitration or Cuen and viscometry technique. Viscometry was used on corn stover, poplar, and wheat straw organosolv studies, while nitration was used on the remaining biomass.

As to be expected, each pretreatment has a different effect on the DP of cellulose and the extent of depolymerization that occurred. A benefit of pretreating biomass is the reduction of the DP of cellulose without extensive degradation providing more reducing ends for the enzymatic hydrolysis [85]. Low pH pretreatments (i.e. dilute acid and SO_2) cause more cellulose depolymerization than the AFEX and ARP pretreatments [70]. The DP of corn stover cellulose decreased by ~60% (from ~7200 to ~2800) after dilute acid and SO_2 pretreatments, while alkaline based pretreatments caused ~6% (AFEX) and

~38% (ARP) reduction in DP [70]. A similar effect occurred to poplar cellulose, where the DP of cellulose was reduced by ~86% and ~20% after acidic and basic pretreatments, respectively [70]. For the mixture of spruce and pine (50:50), as the dilute acid pretreatment severity increased, the cellulose DP decreased from 700 until it reached the leveling-off value of 200 [73]. Furthermore, for ozone (O_3) pretreated samples, the decrease in DP was less than that in the carbon dioxide explosion (CE) and alkaline explosion (AE) pretreated samples. For example, the DP of bagasse cellulose decreased from 925 to 800, 572, and 550 after O_3, CE, and AE pretreatments, respectively [91].

For organosolv pretreatment, in which the biomass was pretreated in a mixture of organic solvents, water, and, HCl as catalyst, the DP of cellulose varied across the different organic solvents used [141]. Ethanol gave the lowest DP, methanol and acetic acid gave comparable DPs, and formic/acetic acid system had the least cellulose depolymerization. The organosolv pretreatments were carried under different reaction conditions as shown in Table 20 [141]. Ethanol organosolv pretreatment caused more depolymerization of cellulose than methanol, formic acid, and acetic acid. The increase in organic solvent preserved reduction in DP of cellulose as shown in the higher DP values in samples 2 and 3 compared to 1, as well as the higher DP of sample 5 compared to sample 4.

Table 20. Change in DP of wheat straw cellulose after various organosolv pretreatment[a] [141].

Sample #	Solvents Used	Solvents Ratio (%v/v)	DP
1	Acetic acid-water	65/35	1594
2	Acetic acid-water	80/20	1763
3	Acetic acid-water	90/10	1952
4	Formic acid-acetic acid-water	20/60/20	2182
5	Formic acid-acetic acid-water	30/60/10	2289
6	Methanol-water	60/40	1519
7	Ethanol-water	60/40	1356

[a] Performed using 0.1% HCl as a catalyst at 85 °C for 4 h with a liquor-to-solid ratio of 20:1 (mL/g)

The chemical and physical changes that occur to the biomass after pretreatment are what dictate efficient enzymatic digestibility. Such changes include: decreasing the DP of cellulose, lignin content, hemicellulose content, lignin-carbohydrate complexes, and cellulose accessibility. It is difficult to assess the effect of these factors individually since more than one change usually occurs during pretreatment. Therefore, these collective contributions provide more enzymatically digestible cellulose. Lower DP improves enzymatic hydrolysis due to two factors: (1) increasing the number of cellulose chain reducing ends and (2) making cellulose more reactive to the enzymes. As the DP of cellulose decreases, the number of reducing ends of cellulose increases, thus allowing for more exoglucanase effective activity [72, 134]. Väljamäe *et al*. showed that increasing the number of endoglucanase-generated chain ends from 0 $\mu mol.g^{-1}$ to 6 $\mu mol.g^{-1}$ in bacterial cellulose, enhanced the exoglucanase hydrolysis of cellulose, which resulted in an increase in the amount of solublized total sugars from 0.1 mM to 0.25 mM [142]. Furthermore, shorter chains allow cellulose to be more amenable to enzymatic deconstruction because they do not form strong hydrogen bonding (i.e., they form weaker networks permitting greater possibility for enzyme access [72, 134]. Depending on the

pretreatment technology used, the resulting pretreated lignocellulosic substrate will contain different amount of reducing chain ends. For example, solids resulting from low pH pretreatments (dilute acid and SO_2,) have higher reducing ends than high pH pretreatments (AFEX and lime) [70].

The study by Puri showed that the enzymatic saccharification increased with reduction in cellulose DP [91]. For instance, the extent of enzyme saccharification increased from 28% for the untreated bagasse to 86%, 78%, and 85% after O_3, CE, and AE pretreatment, respectively. The mixture of spruce and pine samples also showed higher enzymatic saccharification as the cellulose DP decreased [73]. At DP 200, 40% saccharification was achieved as compared to 5% saccharification at DP 400 [73]. Although changes in the structure of cellulose undoubtedly contributed to the reduced recalcitrance observed for these pretreated biomass samples, additional changes in lignin and hemicellulose also contributed as well; and thus future research is needed to determine their relative importance.

2.2.3.3 Effect of Pretreatment on Cellulose Crystallinity

Many studies have examined the effect of pretreatment on the crystallinity of cellulose because it has long been postulated to play a role in its susceptibility to enzyme digestion [143]. Table 21 summarizes the CrI (crystallinity index) of cellulose from various untreated and pretreated biomass after some pretreatment processes, such as ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute sulfuric acid, lime, SO_2, ozone (O_3), carbon dioxide explosion (CE), alkaline explosion (AE), and organosolv.

Table 21. Crystallinity index of several untreated and pretreated biomass cellulose using various pretreatment technologies.

Biomass	Pretreatment	Crystallinity Index (%)
Corn Stover[a] [70]	-	50.3
	Ammonia fiber expansion	36.3
	Ammonia recycled percolation	25.9
	Controlled pH	44.5
	Dilute Acid	52.5
	Lime	56.2
Poplar[a] [70]	-	49.9
	Ammonia fiber expansion	47.9
	Ammonia recycled percolation	49.5
	Controlled pH	54.0
	Dilute acid	50.6
	Lime	54.5
	SO_2	56.5
Bagasse[a] [91]	-	37
	O_3	38
	CO_2 explosion	57
	Alkaline explosion	62
Wheat Straw[a] [91]	-	35
	O_3	34
	CO_2 explosion	56
	Alkaline explosion	53
Eucalyptus regnans[a] [91]	-	37
	O_3	40
	CO_2 explosion	53
Pinus radiata[a] [91]	-	34
	O_3	36
Switchgrass[a] [144]	-	46.1
	Lime	51.9
Loblolly pine[b] [65]	-	62.5
	Dilute acid	69.9

[a]Crystallinity index measured by x-ray diffraction. [b]Crystallinity index measured by solid-state CP/MAS ^{13}C NMR spectroscopy.

As to be expected, each pretreatment has a different effect on the crystallinity of cellulose. Some pretreatment technologies caused reduction in cellulose crystallinity, some showed no effect on crystallinity, and others increased the crystallinity. Low pH pretreatments significantly enhanced biomass crystallinity, while all high pH

pretreatments had less effect and even reduced biomass crystallinity in some instances. It also appears that the effect of the same pretreatment is biomass dependent. For instance, controlled pH pretreatment, (i.e. controlling the pH at near neutral conditions), reduced the crystallinity index of corn stover, while it caused an increase in crystallinity of poplar. In general, dilute acid, lime, CO_2 explosion, alkaline explosion, and SO_2 pretreatments increases cellulose crystallinity due to the fact that amorphous cellulose degrades more easily than the more stable crystalline cellulose during pretreatment. Pretreatments such as AFEX and ARP reduced the crystallinity, suggesting possible decrystallization of cellulose. Furthermore, the resulted crystallinity of the pretreated biomass is dependent on the pretreatment conditions employed. Table 22 shows that cellulose crystallinity increased when the pretreatment severity increased from condition set 1 to 3, causing an enrichment in the crystalline form of cellulose due to the selective hydrolysis of amorphous cellulose during the pretreatment [134].

Table 22. Crystallinity of ethanol organosolv pretreated substrates prepared from lodgepole pine under varied conditions [134].

Condition	T (°C)	t (min)	S (%)	C (%)	CrI (%)
1	170	60	0.76	65	75
2	170	60	1.10	65	78
3	180	60	1.10	65	85

T, temperature (8C); t, time at temperature (min); S, sulfuric acid dosage (% on oven dry chip, w/w); C, concentration of ethanol (%, v/v); CrI, crystallinity index determined by x-ray diffraction.

Jeoh *et al*. has demonstrated the effect of cellulose crystallinity on cellulase accessibility [143]. Amorphous cellulose samples were prepared from avicel and filter paper as follows: cellulose was dissolved in a dimethylsulfoxide-paraformaldehyde solution, and then regenerated by slow addition of the cellulose solution to a solution of

0.2 M sodium alkoxide in methanol/i-propanol (1:1). This procedure has been demonstrated to produce amorphous cellulose without altering the degree of polymerization (DP) and reducing end group concentration of the starting cellulose [143]. The resulted amorphous forms of celluloses were found to be significantly more digestible than the original crystalline forms. For both avicel and filter paper, the extent of cellulose hydrolysis increased from 10% to 80% for crystalline and amorphous forms, respectively [143]. Specifically, the bound cellulase concentrations on the amorphous forms of both filter paper and Avicel were significantly higher than on the crystalline forms (i.e. 0.1 μmoles/g of remaining cellulose for the crystalline form and 1.5 μmoles/g of remaining cellulose for the amorphous form) [143]. The maximum extents of binding on the amorphous forms increased on the order of 15-fold over that of the original forms. The change in crystallinity of the cellulose samples, therefore, may have allowed increased access to cellulase [143]. One could thus infer that the increased access may have contributed to the increased cellulose hydrolysis rates observed.

Zhu *et al*. illustrated the relationship between biomass digestibility and crystallinity (Figure 27). Hybrid poplar was treated with varying amounts of peracetic acid and KOH to generate samples with different crystallinity [145]. The results clearly indicate that the enzymatic digestibility of the biomass increased with decreasing biomass crystallinity, suggesting that amorphous cellulose is more accessible to enzymatic digestibility. The relationship was linear between enzymatic hydrolysis and biomass crystallinity for the 1 h, 10 FPU/g biomass and 72 h, 0.25 FPU/g biomass; while it was not linear for the other two, indicating that the hydrolysis reached completion.

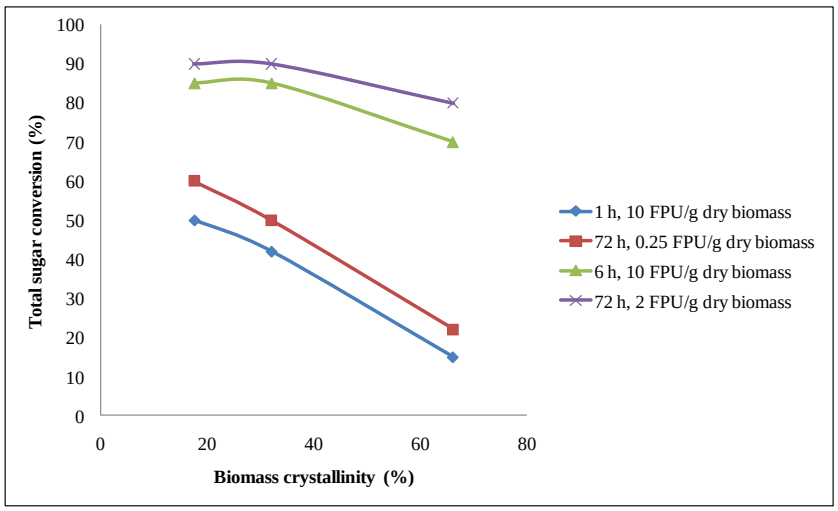


Figure 27. The relationship between crystallinity of poplar and its enzymatic digestibility [145].

2.2.3.4 Effect of Pretreatment on Surface Area and Pore Volume of Biomass

Pore volume of biomass is another physical structural feature influencing the enzymatic digestibility of cellulose. Providing more or larger pores within the biomass matrix makes cellulose more amenable to cellulase. Cellulase is typically from ca. 24 to 77 Å in diameter, with mean of 59 Å [146]. Pretreatment of biomass has been shown to increase the specific surface area of biomass; thus allowing for enhanced conversion of cellulose to glucose by cellulase. The accepted explanation for the increase in surface area is that upon removal of cell wall components (mainly lignin), surface openings or internal slits, voids, or spaces are created; therefore increasing the pore volume [145, 147, 148]. The effect of various pretreatment technologies on different biomass has been extensively studied [149-151]. Grethlein studied the effect of acid pretreatment on the specific surface area of mixed hardwood (90% birch and 10% maple), poplar, and white pine (Table 23) [149]. For both hardwood and softwood, acid pretreatment caused an increase in the specific surface area of the biomass, which in turn allows for more pore

volume accessible to the enzyme. Specific surface area is also susceptible to pretreatment severity. As shown in Table 23, the specific surface area of mixed hardwood increased as the pretreatment severity from 10.5 to 140 m^2/g.

Table 23. Effect of acid pretreatment on the specific surface area of several biomass [149].

Substrate	Pretreatment conditions			SSA_{51}[a] (m^2/g)
	Temp. (°C)	Acid (%)	Time	
Mixed hardwood[b]	-	-	-	10.5
	100	1	5 hr	37.1
	180	1	7.8 sec	47.6
	200	1	7.8 sec	112
	220	1	7.8 sec	140
Poplar	-	-	-	6.5
	200	0.41	6 sec	88
White pine	-	-	-	13.7
	200	1	7 sec	80

[a] Specific surface area available to a 51 Å solute; [b] 90% birch and 10% maple.

A study by Gharpuwy *et al*., showed the effect of three pretreatments (caustic, peracetic acid, and ethylene glycol) on the specific surface area of wheat straw (Table 24) [152]. The caustic pretreatment was performed using 1% (0.25N) NaOH solution. One hundred grams of wheat straw were mixed with l000 mL of the NaOH solution. This mixture was autoclaved for 2 h at 129 °C and 2.57 atm (23 psig). In peracetic acid pretreatment [1:1 (v/v) acetic anhydride and 35% hydrogen peroxide], 100 g of wheat straw were boiled with l000 mL of peracetic acid at 100°C for 30 min. In the ethylene glycol pretreatment, 48 g of wheat were mixed with 600 mL of ethylene glycol and 13 mL of concentrated HCl. This mixture was autoclaved at 129 °C and 23 psig for 1 h. All of the chemical pretreatments led to an increased surface area. The conclusion was these

increases might be due to an inflation of the lignocellulosic structure upon removal of the lignin glue by the chemical action [152].

Table 24. Effect of caustic, peracetic acid, and ethylene glycol pretreatment on the specific surface area of wheat straw [152].

Pretreatment	Specific surface area (m^2/g)	Lignin (%)	Maximum enzymatic conversion (%)
-	0.64	11.53	5.5
Caustic	1.7	5.29	62.0
Peracetic acid	1.7	2.60	54.5
Ethylene glycol	2.9	3.27	54.5

Another study by Ishizawa *et al*. evaluated whether porosity was one of the factors governing the overall enzymatic digestibility of the cellulose in dilute-acid-pretreated corn stover [147]. Corn stover was subjected to dilute H_2SO_4 pretreatment using a fixed residence time of ~1 min at temperatures ranging from 180 to 200 °C, solid loadings between 25% and 35% (w/w), and acid loadings of 0.03-0.06 g of acid/g of dry biomass. All of the pretreated samples showed higher pore volumes than untreated corn stover, particularly in the range from 10 to 100 Å, resulting in higher enzymatic conversion. Table 25 illustrates the effect of dilute acid pretreatment on the pore volume of corn stover. The results clearly shows that the accessible pore volume increased; being highest in pretreated corn stover (PCS) 2, which showed best enzymatic hydrolysis.

Table 25. Accessible pore volume of untreated and dilute acid pretreated corn stover [147].

Probe diameter (Å)	Accessible volume (ml/g)		
	UCS[a]	PCS 1[b]	PCS 2[c]
8	0.9	0.9	1.4
18	0.5	1.0	1.2
32	0.4	0.8	1.2
90	0.1	0.1	0.7
100	0	0	0.5

[a] Untreated corn stover; [b] Pretreated corn stover resulted with 72.5% enzymatic conversion; [c] Pretreated corn stover resulted with 96.3% enzymatic conversion.

CHAPTER 3

EXPERIMENTAL MATERIALS AND PROCEDURES

3.1 Materials

3.1.1 Chemicals

All chemicals were purchases either from Sigma-Aldrich (St. Louis, MO, USA) or VWR (West Chester, PA, USA), and used as received. All gases were purchased from Airgas (Radnor Township, PA, USA).

3.1.2 Biomass Preparation

3.1.2.1 *Buddleja davidii* Samples for Characterization Experiment

Several stems and foliar and floral parts of *B. davidii* was harvested from a single plant from a domestic garden in southern England in September 2007 and divided into three main constituents: leaves, bark, and stem. The samples were air-dried and prepared as follows: stems were manually debarked with a knife, chipped, and milled to pass a 0.40 mm screen; the leaves and bark were ground using a pestle and mortar. *B. davidii* was also harvested from plants from the Georgia Institute of Technology, Atlanta, GA, in September 2008.

3.1.2.2 *Buddleja davidii* for Ethanol Organosolv Pretreatment Experiment

Buddleja davidii stems (approximately diameter 3 cm) were manually debarked with a knife, air-dried in the lab to approximately 15% moisture content (oven dry (od)

basis), and milled using a Wiley mill to pass a 5 mm screen. The milled, air-dried feedstock was stored in a freezer at -5 °C.

3.2 Experimental Procedures

3.2.1 Dichloromethane (DCM) Extraction

The extractives contents were measured according to the TAPPI method T 204 cm-97, in which samples were Soxhlet extracted with dichloromethane (DCM) (Figure 28), and the extractive content was calculated gravimetrically [153].

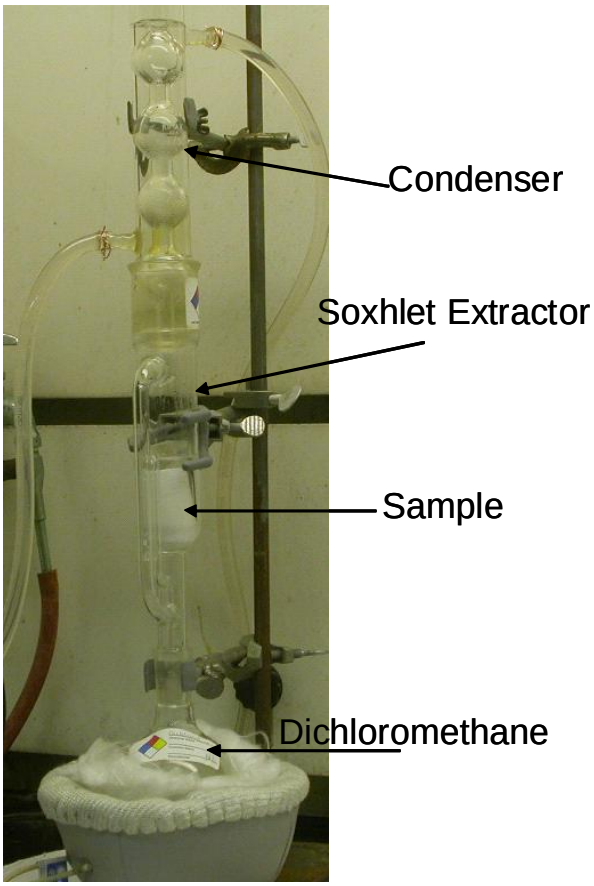


Figure 28. Soxhlet extractor used for dichloromethane (DCM) extraction of *Buddleja davidii* stems, bark, and leaves.

3.2.2 Holocellulose Pulping

The holocellulose pulping was conducted according to modified literature method [154, 155]. The DCM-extracted stem material (9.00 g dry-weight) was dispersed into 750 ml of de-ionized water. Then glacial acetic acid (6.00 mL) and sodium chlorite (6.00 g, $6.63x10^{-2}$ moles) were added to the mixture, which was poured in a plastic pouch and sealed. The mixture was heated in a shaking water bath at 70 °C for 2 hours. After the 2 hours, another batch of glacial acetic acid and sodium chlorite were added, and the mixture was heated in the shaking water bath for 2 more hours. Finally, the solid residue (holocellulose) was filtered out and washed thoroughly with de-ionized water. The whole process was repeated until the holocellulose turned white, and the % Klason lignin was very low (<2%).

3.2.3 Isolation of Cellulose, Hemicellulose, and Milled Wood Lignin

Cellulose was isolated by refluxing a holocellulose sample (2.00 g dry weight) in 2.5 M HCl (200.00 mL) for 4 h, according to the literature method [156]. The cellulose was collected by filtration and allowed to dry.

Hemicellulose was extracted from the holocellulose according to the method described by Jacobs and Dahlman [59]. Briefly, the holocellulose was treated with potassium hydroxide, the cellulose was removed by filtration, and the hemicellulose was precipitated in an ethanol solution containing 30% acetic acid. The hemicellulose was then isolated by centrifugation, allowed to air-dry, and stored in a vial at room temperature.

The methods described by Guerra *et al*. [157], Holtman *et al*. [158], and Sannigrahi *et al*. [65], were used to isolate and purify milled wood lignin (MWL). The *B.*

davidii stems were milled using a Wiley mill to pass through a 0.50 mm screen and 50 g ground wood was treated with 1:2 (v/v) ethanol: benzene for 24 h in a Soxhlet apparatus followed by a 24 h Soxhlet treatment with ethanol to remove extractives. The extractive-free wood was dried, transferred to a porcelain jar and ground in a rotary ball mill for 130 h. Porcelain balls of three different sizes (total weight of 1,000 g) and 200 mL toluene were added to the wood in the jar prior to ball milling. The ball milled sample was dried to remove toluene and extracted twice with p-dioxane–water solution (96% v/v) in a shaker at 190 rpm for 24 h in the dark. The p-dioxane–water extracts were combined and solvent volume was reduced to 100 mL using a rotary evaporator. This solution was added dropwise to DI water (200 mL) while stirring and then freeze dried. The freeze-dried crude MWL was dissolved in 90% acetic acid (20 mL) and precipitated in DI water (400 mL). The solution was centrifuged and the solid material was dissolved in 1,2 dichloroethane/ethanol (10 mL, 2:1 v/v) and precipitated in diethyl ether (200 mL). The solution was centrifuged and the solid material washed with petroleum ether (2×100 mL). The MWL sample obtained was air dried and kept overnight in a vacuum oven (101.6 kPa, 40°C) prior to NMR analysis.

3.2.4 Ethanol Organosolv Pretreatment

The ethanol organosolv process described by Pan *et al.* was followed [129, 130, 132]. A 20.00 g (od weight) sample of the biomass was treated in aqueous ethanol (volumetric ratio of 1:1, 1:1.5, or 1:4 water-to-ethanol), with sulfuric acid as catalyst, in a 1 L pressure reactor with temperature controller (Parr Instrument Company, Moline, IL). Pretreatment conditions are summarized in Table 26. The temperature ramp rate is about 3.5 °C/min.

Table 26. Process conditions for the ethanol organosolv pretreatment of *Buddleja davidii*.

Condition	Temperature (°C)	Time (min)	Sulfuric Acid (%, w/w oven-dried wood)	Ethanol Concentration (%, v/v)
A	180	60	1.25	50
B	180	40	1.75	50
C	195	60	1.50	65
D	180	80	1.25	50
E	170	40	1.50	65
F	195	60	2.00	65

Immediately after the pretreatment time, the pressure was released slowly and cautiously and the effluent was sampled to determine furfural and 5-hydroxylmethyl furfural (HMF) contents as these are known inhibitors of downstream fermentation processes [136]. Then, the assembly was allowed to cool to about 80°C before the reactor was opened. Afterwards, the pretreated wood was washed thoroughly with 60 °C aqueous ethanol solution having the same concentration as the cooking liquor. The washed biomass (i.e., solid fraction) was allowed to dry for carbohydrates and lignin analysis. The effluent and ethanol washes were combined and mixed with three volumes of water to precipitate the dissolved lignin, which is defined as ethanol organosolv lignin (EOL). The lignin was filtered out, and the filtrate (water-soluble fraction), which mainly contains soluble lignin, monomeric and oligomeric hemicelluloses, was retained for compositional analysis.

3.3 Analytical Procedures

3.3.1 Carbohydrates and Lignin Content

The carbohydrate profile and lignin content were measured as previously described in literature [65, 159]. The untreated and pretreated wood (0.175 g dry weight) was hydrolyzed with 72% sulfuric acid for 1 h, diluted to 3% sulfuric acid with water, and then autoclaved at 121 °C for 1 h. Afterward, the samples were filtered, and the residue was dried and weighed to give the Klason lignin content. The acid-soluble lignin in the filtrate was measured from the absorbance at 205 nm, and the carbohydrates were quantified using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The system used was Dionex ICS-3000 ion chromatography with CarboPactm PA-1 column. The column was set at 23 ºC and eluent A was 100% DI water (18 MΩ-cm) and the eluent B was 200 mM NaOH. The flow rate was 0.3 ml/min.

3.3.2 Ash Analysis

Inductively coupled plasma (ICP) analysis was performed on all three samples (stem, leaves, and bark) for the analysis of trace inorganic elements [160]. The ash content was measured by heating the samples at 525 °C in a furnace.

3.3.3 Enzymatic Hydrolysis

The commercial enzymes from Sigma-Aldrich, cellulase (Celluclast 1.5 L) and β-glucosidase (Novozym 188), were used in this study as received. Enzyme activity was measured according to the laboratory analytical procedure (LAP) provided by NREL

[161]. The enzymatic hydrolysis was carried out on the pretreated *B. davidii* wood containing 1% cellulose w/w in 100.0 mL of 50 mM acetate buffer (pH 4.8). The cellulase to β-glucosidase ratio was 1 FPU:2 IU, with loadings of 20 FPU/g cellulose and 40 IU/g cellulose [129, 132]. The reaction mixture was incubated at 150 rpm and 45 °C in a rotary shaker for 72 h. Samples were taken at 0, 2, 4, 6, 24, 48, and 72 h. The glucose content was measured using a Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD) equipped with a SIL-10AD autosampler, an LC-10AD pump, an Alltech Prevail carbohydrate ES column (Grace, Deerfield, IL), and an evaporative light scattering detector (PL-ELSD 1000) (Polymer Laboratories, Amherst, MA). Samples (50 μL) were filtered using a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter and eluted at 1 mL/min, with 75% v/v acetonitrile as the mobile phase.

3.3.4 NMR Spectroscopy

All NMR analyses were carried out using a 400 MHz DMX Bruker spectrometer (Billerica, MA, USA), operating at 100.59 MHz for ^{13}C.

3.3.4.1 Solid-State CP/MAS ^{13}C NMR Characterization of Cellulose

The solid-state CP/MAS ^{13}C NMR experiment and the spectral fitting were performed as previously described [64, 65]. The extracted cellulose samples (~30% moisture) were packed in 4 mm ZrO_2 rotors fitted with Kel-F caps and spun at 8 kHz. The spectra were acquired with 8129 scans, 25.0 Hz line broadening, zero filling of 2048, and the line-fitting analysis was done using NUTS NMR Data Processing software (Acorn NMR, Inc.).

Solid state CP/MAS ^{13}C NMR is a technique that has been used to determine the relative intensities of these various cellulose structures. The technique is based on a line-shape spectral fitting analysis of the C-4 region of the solid-state CP/MAS ^{13}C NMR spectra, where Lorentzian lines are used to fit the I_α and I_β signals, and Gaussian lines are used to fit the signals from the other remaining structures (*para*-crystalline, accessible fibril surfaces, and inaccessible fibril surfaces). The chemical shift and width of each line used to fit the spectra are based on literature values. The intensity of each peak is adjusted/iterated to achieve best spectral fitting.

3.3.4.2 Quantitative ^{13}C NMR Characterization of Lignin

Quantitative ^{13}C NMR spectrum was acquired using DMSO-d_6 (500 μL) as solvent for lignin (80 mg), with an inverse gated decoupling sequence, 90° pulse angle, 12 s pulse delay, and 12288 scans. The acquisition temperature is 50 °C with 10.0 Hz line broadening, and no zero filling.

3.3.4.3 Quantitative ^{31}P NMR Characterization of Lignin

As previously described, quantitative ^{31}P NMR were acquired on dry lignin (~25 mg) dissolved in a solvent mixture consisting of 1.6:1 (v/v) anhydrous pyridine/deuterated chloroform with chromium (III) acetylacetonate (~3.6 mg/mL) as a relaxation agent and cyclohexanol (~4.0 mg/mL) as an internal standard [162, 163]. The dissolved lignin is then derivatized with 100 μL of TMDP. The spectrum was acquired using an inverse gated decoupling pulse sequence, 90° pulse angle, 25 s pulse delay, 200 scans, no zero filling, 4.0 Hz line broadening, and at room temperature.

3.3.4.4 DEPT-135 ^{13}C NMR Characterization of Lignin

DEPT spectra were acquired using 135° pulse angle, 3-s pulse delay, and 8192 scans. The acquisition temperature is 50 °C with no zero filling and 5.0 Hz line broadening.

3.3.4.5 Qualitative ^{13}C-^{1}H 2D HSQC Characterization of Lignin

Conditions for the HSQC analysis were as follows: 13-ppm spectra width in F2 (^{1}H) dimension with 1024 data points (95.9-ms acquisition time), 210-ppm spectra width in F1 (^{13}C) dimension with 256 data points (6.1-ms acquisition time); a 3-s pulse delay; a $^{1}J_{C\text{-}H}$ of 145 Hz; and 32 scans. The central solvent peak (δ_C 39.5 ppm; δ_H 2.5 ppm) was used for chemical shifts calibration [11, 164]. The acquisition temperature is 50 °C. Spectra processing used a typical squared sine apodization in F2 and F1. NMR data were processed using the TopSpin 2.1 software (Bruker BioSpin).

3.3.5 Gel Permeation Chromatography Analysis of Cellulose

The number-average molecular weight ($\overline{M}_n$) and the weight-average molecular weight ($\overline{M}_w$) were determined by gel permeation chromatography (GPC) after tricarbanilation of cellulose [82]. Cellulose sample (15 mg) was dried over P_2O_5 under vacuum (762 mm of Hg) at 40 °C for 24 h and then was derivatized by adding anhydrous pyridine (4.00 mL) and phenyl isocyanate (0.50 mL, 4.62×10^{-3} moles). The sealed reaction flask was kept at 65 °C with stirring until the cellulose was completely dissolved. Afterwards, the solution was cooled and methanol (1.00 mL) was added to the reaction mixture to eliminate the unreacted phenyl isocyanate. The mixture was then

poured into a 3:7 water-methanol mixture (100 mL), and the precipitated cellulose tricarbanilate was removed by centrifugation. The derivatized cellulose was purified by repeated washing with water-methanol (3 x 100 mL) followed by water (2 x 100 mL). Finally, the cellulose was freeze-dried and vacuum dried for GPC analysis.

The cellulose tricarbanilate was analyzed for molecular weight and molecular weight distribution using a Hewlett Packard 1090 series HPLC system consisting of an auto-sampler, a UV detector, and three columns of Styragel HR1, HR3, and HR4 (Waters Inc., USA) linked in series using tetrahydrofuran (THF) as the eluent. The derivatized cellulose was dissolved in THF (1 mg/mL), and the solution was filtered through a 0.45 µm filter. Then the filtered solution (20 µl) was injected into the HPLC system and was detected using the UV detector at 236 nm. Standard narrow polystyrene samples were used in order to construct a calibration curve. Data were collected with Agilent ChemStation Rev. A.10.01 and analyzed with Agilent GPC Addon Rev. A.02.02 software. The values $\overline{M}_n$ and $\overline{M}_w$ were calculated using the GPC software, and the weight-average degree of polymerization (DP_w) was obtained by dividing $\overline{M}_w$ by 519, the molecular weight of the cellulose tricarbanilate monomer.

3.3.6 Gel Permeation Chromatography Analysis of Lignin

The $\overline{M}_n$ and $\overline{M}_w$ of isolated lignin were determined by GPC after acetylation of lignin to allow dissolution in THF [165]. About 20 mg of lignin was dissolved in a 1:1 acetic anhydride-pyridine mixture (1.00 mL) and kept for 24 h at room temperature. Ethanol (25.00 mL) was added to the reaction mixture, left for 30 minutes, and then removed with a rotary evaporator. The addition and removal of ethanol was repeated 7

times to insure complete removal of acetic acid and pyridine from the sample. Afterwards, the acetylated lignin was dissolved in chloroform (2.00 mL) and added drop wise to diethyl ether (100.00 mL) followed by centrifugation. The precipitate was washed three times with ether and then dried under vacuum (762 mm of Hg) at 40 °C for 24 h. GPC analysis for lignin was performed the same way as described for cellulose, except that the UV detector was set at 280 nm.

3.3.7 Gel Permeation Chromatography Analysis of EOL

The number-average molecular weight ($\overline{M}_n$) and the weight-average molecular weight ($\overline{M}_w$) were determined by GPC after acetylation of lignin [166]. The lignin samples (~20 mg) were dissolved in a 1:1 acetic anhydride/ pyridine mixture (1.00 mL) and kept for 24 h at room temperature. The solvent mixture was concentrated under reduced pressure at 50 °C, dissolved in chloroform (50 mL), washed with water (3 x 20 mL), and dried over anhydrous $MgSO_4$. The mixture was then filtered, and the chloroform was removed with a rotary evaporator. The dried acetylated lignin was analyzed for molecular-weight properties with an Agilent 1200 series GPC system using four columns Styragel HR1, HR2, HR4, and HR6 (Waters, Inc., Milford, MA) linked in series and tetrahydrofuran (THF) as the eluent. Acetylated lignin was dissolved in THF (1.0 mg/mL), filtered through a 0.45 μm filter, injected (20 μL) into the GPC system, and detected using a UV detector set at 270 nm. The flow rate was 1.00 mL/min. Standard narrow polystyrene samples were used to establish a calibration curve. Data were collected and analyzed with WinGPC Unity software (version 7.2.1, Polymer Standards Service USA, Inc., Warwick, RI).

3.3.8 Error Analysis

For the characterization studies of *B. davidii*, the error in the analytical measurements was determined from duplicate runs of each analysis. The error margins associated with the carbohydrate measurements were ±0.01% to ±2.83%, lignin measurements were ±0.08% to ±0.73%, furfural and HMF measurements were ±0.02%, ash and extractives analyses were ±0.01% to ±0.07%. The standard errors associated with the quantitative ^{13}C and ^{31}P NMR analyses were 3.0 and 1.2%, respectively.

CHAPTER 4

BIOMASS CHARACTERIZATION OF *BUDDLEJA DAVIDII*: A POTENTIAL FEEDSTOCK FOR BIOFUEL PRODUCTION[1]

4.1 Introduction

The demand for energy is increasing every year and is expected to grow by more than 50% by 2025, but the finite amount of fossil fuel available will be insufficient to satisfy this increase [1, 13]. These demands and the recognition that society needs to develop sustainable energy technologies have focused the attention of many researchers on the development of alternative and renewable sources of energy, especially transportation fuels.

A promising sustainable source of energy is biofuels, including bioethanol from a variety of bioresources. Currently, bioethanol is produced primarily from the fermentation of sucrose and starches, which is frequently referred to as first-generation bioethanol [2, 13]. In the United States and Brazil, this is accomplished using primarily corn starch and sugar cane respectively [1, 167]. The need to broaden this bioresource base has been acknowledged by many. Second-generation bioethanol provides an alternative technology that utilizes lignocellulosic materials such as wood agricultural or forest residues to produce biofuels. The benefits of this approach are numerous including:

[1] This manuscript was accepted for publication in Journal of Agricultural and Food Chemistry, 2009. It is entitled as "Biomass Characterization of *Buddleja davidii*: A Potential Feedstock for Biofuel Production". The other authors are Poulomi Sannigrahi, Yunqiao Pu, and Arthur J. Ragauskas from the Institute of Paper Science and Technology and School of Chemistry and Biochemistry at Georgia Institute of Technology and Michael Ray and Richard J. Murphy, from the Division of Biology at Imperial College London, UK.

1) they do not directly compete with food, 2) have a greater net energy generation per area of land, and 3) can be grown on non-agricultural lands.

Buddleja davidii Franch. is a shrub that originated in China, but has been naturalized in different parts of the world, including parts of the U.S. and Europe [24]. This plant has several attractive agro-energy features that make it an interesting potential source for biofuels. *B. davidii* exhibits a very wide range of growth habitat and is well adapted to growing in any soil conditions [26]. Moreover, the plant is perennial, has favorable growth dimensions (up to 3-5 m high), and has very few pests or diseases [26]. This suggests *B. davidii* could be a valuable future agro-energy crop for second-generation ethanol production although, to date, no attempts have been made to 'domesticate' it as a crop species.

The process of producing bioethanol from lignocellulosic materials involves several steps (Figure 29) [7]. Briefly, the material is pretreated to alter its structure so that the biomass matrix can be more accessible to enzymatic hydrolysis [111]. During pretreatment, lignin is partially removed from the biomass, most of the hemicelluloses are hydrolyzed, and the crystallinity of cellulose is disrupted to a certain degree. Enzymes, such as cellulases, can then be used to hydrolyze the cellulose to glucose, and glucose is then commonly fermented to ethanol [7].

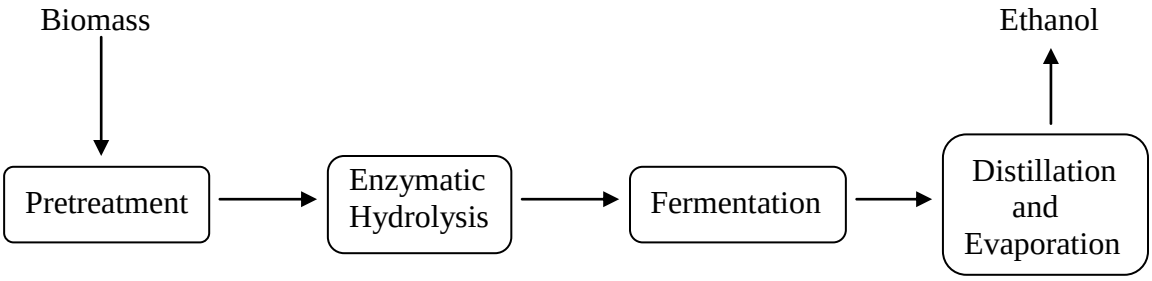


Figure 29. The process of a typical ethanol production scheme from biomass.

Biomass characterization needs to be performed in order to understand the basic composition of the biomass and how its fundamental structure can be altered during pretreatment to optimize the release of C6 and C5 sugars for fermentation. This study focuses on the determination of ash, extractives, lignin, hemicellulose, and cellulose content in *B. davidii*, as well as, detailed elucidation of the chemical structures of both lignin and cellulose by NMR. The results will be used in future work to design the processes to convert *B. davidii* biomass into bioethanol.

4.2 Experimental Section

4.2.1 Sample Preparation

Samples were prepared as described in Chapter 3 (3.1.2.1 *Buddleja davidii* Samples for Characterization Experiment). All the results from the analyses were calculated based on the oven dry weight of biomass that was determined by measuring the moisture content using a moisture analyzer. The analyses that include standard deviations were done in two replicates.

4.2.2 Ash and Extractive Contents

The extractives contents in the stem, bark, and leaves were measured as described in Chapter 3 (3.2.1 Dichloromethane (DCM) Extraction). Ash analysis was performed as described in chapter 3 (3.3.2 Ash Analysis).

4.2.3 Tannin Content

DCM-extracted bark (2.00 g dry-weight) was Soxhlet extracted with methanol-water mixture (75% v/v) for 24 h, and then freeze-dried to recover the tannins [168].

4.2.4 Heating value

The heating value of the leaves, bark, and stem were measured for combustion in an adiabatic oxygen bomb calorimeter according to the TAPPI method T 684 om-06 [169].

4.2.5 GC/MS Analysis

The DCM extractives were concentrated under a stream of nitrogen at room temperature and then derivatized using MSTFA (N-Methyl-N-trimethylsilytrifluoroacetamide). The prepared sample was analyzed employing GC/MS with splitless injection. The instrument used was a Hewlett-Packard 5890 II GC equipped with Hewlett-Packard 5971A Mass Selective Detector. A 0.25 mm x 60 m DB-5 fused silica capillary column with a 25 μm coating stationary phase was used for the chromatographic separations. The GC conditions were: initial temperature 150 °C; initial time 5 min.; rate 15 °C / min.; final temperature 280 °C and final time 25 min; inject port temperature 250 °C. The mass detector was operated by using the following conditions:

EI model; 70 eV; filament on delay time 8 min.; mass scan range: 45 to 650 mu. Quantification of individual components was based on the total ion peak area. GC response factor of each individual compound was assumed to be one for all calculations.

4.2.6 Carbohydrate and Lignin Composition

Carbohydrate profiles and lignin (Klason and acid soluble) content in the stem, bark, and leaves were determined as described in Chapter 3 (3.3.1 Carbohydrates and Lignin Content). Uronic acid content was determined using the colorimetric technique described by Scott [170].

4.2.7 Holocellulose Pulping

The holocellulose pulping was conducted as described in Chapter 3 (3.2.2 Holocellulose Pulping).

4.2.8 Cellulose Analysis

Cellulose was isolated as described in Chapter 3 (3.2.3 Isolation of Cellulose, Hemicellulose, and Milled Wood Lignin). The solid-state CP/MAS ^{13}C NMR experiment and the spectral fitting were performed as described in Chapter 3 (3.3.4.1 Solid-State CP/MAS ^{13}C NMR Characterization of Cellulose).

4.2.9 Hemicellulose Isolation

Hemicellulose was extracted from the holocellulose as described in Chapter 3 (3.2.3 Isolation of Cellulose, Hemicellulose, and Milled Wood Lignin).

4.2.10 Lignin Structural Analysis

Milled wood lignin (MWL) was isolated as described in Chapter 3 (3.2.3 Isolation of Cellulose, Hemicellulose, and Milled Wood Lignin). The elemental analysis (C, H, and O) was performed by Atlantic Microlab, Inc., Norcross, GA. Three NMR experiments were performed: distortionless enhancement by polarization transfer (DEPT), quantitative ^{13}C NMR and ^{31}P NMR spectroscopy as described in Chapter 3 (3.3.4 NMR Spectroscopy).

4.2.11 Gel Permeation Chromatography Analysis of Cellulose

The number-average molecular weight ($\overline{M}_n$) and the weight-average molecular weight ($\overline{M}_w$) were determined by GPC as described in Chapter 3 (3.3.5 Gel Permeation Chromatography Analysis of Cellulose).

4.2.12 Gel Permeation Chromatography Analysis of Lignin

The $\overline{M}_n$ and $\overline{M}_w$ of isolated lignin were determined by GPC as described in Chapter 3 (3.3.6 Gel Permeation Chromatography Analysis of Lignin).

4.3 Results and Discussion

4.3.1 Ash and Extractive Analyses

The ash and extractive contents of the leaves, bark, and bark-free stems are summarized in Table 27. The leaves had a higher ash and extractive content than the bark and the stem because the leaves contain more inorganic elements as shown in Table 28.

The ICP values have percent Relative Standard Deviation (%RSD) ranged from 1% - 20%. The ICP analysis showed that the relative concentration of the elements in this plant was K > Ca > P > S > Mg > Na > Fe > Si > Sr > B > Mn > Cd. The distribution of the elements between the leaves, bark, and stem differed depending on the element, but as would be expected, the mineral elements were found predominantly in the metabolically active parts of the plant, i.e. the leaves and bark. For subsequent bioprocessing of *B. davidii* into a biofuel, these non-processed elements represent a waste stream that will need to be addressed in an environmentally acceptable manner.

Table 27. Ash and extractive contents in the leaves, bark, and stem of native *Buddleja davidii*.

	Leaves	Bark	Stem
Ash (%)	6.30 ± 0.07	4.75 ± 0.07	0.73 ± 0.06
Extractives (%)	3.22 ± 0.07	2.83 ± 0.04	0.30 ± 0.01

Table 28. Inorganic trace elements profile for the leaves, bark, and stem of native *Buddleja davidii*.

Samples/Elements[a]	K	Ca	P	S	Mg	Na	Fe	Si	Sr	B	Mn	Cd
Leaves	7855	16050	2865	2260	853	143	117	83	48	41	43	0.2
Bark	16400	5705	1850	790	1452	434	48	30	32	20	17	0.3
Stem	2470	1175	541	268	174	128	14	9	7	6	4	0.2

[a] Elements values are presented as mg of element/kg of sample.

The heating values of the biomass constituents were measured in order to assess total energy values (Table 29). The heating value of *B. davidii* has been compared with other heating values from various wood species. Table 30 shows that *B. davidii* has a heating value that compares favorably with those for softwoods and hardwoods. According to White [171], the heating value of wood is dependent on the amount of lignin and extractives, it increases with higher lignin and extractive contents. Overall, *B.*

davidii stem wood has a heating value more akin to the higher values of softwoods than hardwoods, probably due to its relatively high lignin content at 30% on a dry weight basis.

Table 29. Summary of the heating values of the leaves, bark, and stem of native *Buddleja davidii*.

Samples	Heating Value (MJ/Kg)
Leaves	19.77 ± 0.15
Bark	21.31 ± 0.02
Stem	20.32 ± 0.22

Table 30. Heating values, and lignin and extractive contents of *Buddleja davidii* and different wood species.

Wood Species	Klason Lignin (%)	Extractives (%)	Heating Values (MJ/Kg)	
			Unextracted Wood	Extractive-Free Wood
Buddleja davidii	30	0.30	20.32 ± 0.22	20.13 ± 0.05
Softwoods [171]				
Engelmann spruce	27	2.40	20.14	19.95
Redwood	34	8.20	21.23	20.81
Hardwoods [171]				
Yellow-poplar	21	3.80	19.65	19.46
Basswood	18	6.70	20.00	19.30

One of the main operations of the future biorefinery, besides producing biofuels, would be to extract high-value chemicals that may already be present in or easily synthesized from the biomass [1]. For instance, the tannin in the bark has been used for antioxidants [3]. This plant has 9.45±1.17 % tannin in the bark. The foliage and flowers of *Buddleja* species are also known to contain anti-inflammatory compounds (flavonoids, carotenoids), antioxidant compounds (flavonoids and phenylethanoids), and antibacterial compounds (e.g. verbascoside against *Staphylococcus aureus*) [31, 172], which, though

not investigated here, may also offer potentially high-value chemicals for extraction in biorefining. After extracting such high-value chemicals, the leaves and bark may be useful for combustion for heat and power production, composted for horticultural use, or investigated further as a potential animal feed (assuming protein, energy, and mineral contents are appropriate). They may also enter further processing for bioethanol production. To identify other potential chemicals in *B. davidii*, GC-MS analysis was performed on the DCM extracts. The stem wood had a low content of extractives (Table 27 and Table 31). However, the extractives content was approximately 10 times higher in the leaves and bark than in the stem. This was also reflected in substantially elevated levels of aromatics, alkanes, fatty acids, alcohols, and sterols in the leaves/bark than in the stem.

Table 31. Evaluation of dichloromethane (DCM) extracts by GC-MS from the leaves, bark, and stem of native *Buddleja davidii*.

Compounds[a]/Samples	Leaves	Bark	Stem
Aromatic Compounds	**13.49**	**171.97**	**4.28**
4-hydroxyphenylethanol	13.49	7.44	4.28
Benzaldehyde	-	29.63	-
vanillic acid	-	73.16	-
α-hydroxy- α -(4-hydroxy-3-methoxy)phenylacetic acid	-	35.43	-
Isopimaradiene	-	13.50	-
Ferulic acid	-	12.81	-
Alkanes	**1000.76**	**143.80**	**0**
C23	51.90	-	-
C25	56.78	-	-
C27	88.92	-	-
C29	594.25	107.93	-
C31	208.91	35.87	-
Fatty Acids	**1410.95**	**2583.43**	**187.12**
C14:COOH	28.72	-	-
C16:COOH	365.24	275.52	95.20
C18:COOH	42.56	88.01	14.96
C19:COOH	-	-	2.20
C20:COOH	48.00	450.36	10.33
C21:COOH	-	-	2.79
C22:COOH	64.62	802.44	9.14
C23:COOH	-	12.51	5.23
C24:COOH	51.63	397.60	4.94
C26:COOH	17.47	35.97	-
C28:COOH	18.92	-	-
C30:COOH	28.95	-	-
9,12-Octadecadienoic acid	127.15	286.33	28.82
oleic acid	-	81.69	-
Linolenic acid	617.69	116.75	7.86
11-Eicosaenoic acid	-	14.69	-
2,3-dihydroxy-hexadecanoic acid	-	21.56	3.33
Azelaic acid	-	-	2.32
Alcohols	**336.72**	**26.20**	**7.40**
C22:OH	25.46	15.52	5.99
C24:OH	232.29	10.68	-
C28:OH	13.71	-	1.41
C30:OH	39.17	-	-
C32:OH	26.09	-	-
Sterols	**159.41**	**99.74**	**69.91**
Stigmasterol	36.66	52.37	26.14
Sitosterol	122.75	47.37	41.60
Ergosterol	-	-	2.17

[a] Compounds values are presented as µg of compound/g of sample.

4.3.2 Biomass Composition

The cell wall macromolecular biomass composition of *B. davidii* is detailed in Table 32. *B. davidii* had a lower cellulose content and higher hemicellulose and lignin contents compared with some common softwood and hardwood species [3]. The relatively high lignin content has potential implications for the effectiveness of pretreatment processes in releasing carbohydrates for fermentation, and the high hemicellulose content indicates a significant amount of C5 as well as C6 sugars available for conversion to bioethanol. The carbohydrate profile in Table 33 indicates a relatively low glucose content (39%) for *B. davidii* stem material [3] compared with other wood species, and a glucose to xylose ratio of 1.8:1.0. Given the relatively high hemicellulose content, micro-organisms capable of fermenting C6 and C5 sugars should appear to be desirable for the efficient conversion of this bioresource to ethanol. As expected for the bark, the lignin content was high (56%); the carbohydrate composition exhibited a few differences from the stem but glucose and xylose were the dominant sugars present (respectively 17% and 11%) (Table 33). The leaves contained ~31% Klason lignin, no acid-soluble lignin, and low carbohydrate content with approximately 11% glucose.

Table 32. Cell wall macromolecule composition of *Buddleja davidii* and other wood species, including lignin, cellulose and hemicelluloses.

	Wood Macromolecules[a]		
Wood Species	Lignin (%)	Cellulose (%)	Hemicellulose (%)
Buddleja davidii	30	35	34
Softwoods [3]			
Picea glauca	27	41	31
Pinus strobus	29	41	27
Hardwoods [3]			
Populus tremuloides	21	48	27
Betula papyrifera	19	42	38

[a] All samples were analyzed extractive-free

Table 33. Carbohydrate profile and lignin content in native *Buddleja davidii* stem, bark, and leaves.

	Relative Carbohydrate Composition[a] (mass %)						Lignin[b] (%)	
	Ara	Gal	Glu	Xyl	Man	4-O-MeGlcA and GalA	Klason	Acid-Soluble
Stem	0.48 ± 0.01	0.93 ± 0.02	38.88 ± 1.22	21.69 ± 0.65	3.04 ± 0.11	4.25 ± 0.21	29.68 ± 0.15	0.52 ± 0.01
Bark	5.40 ± 0.31	2.45 ± 0.47	16.84 ± 2.30	11.06 ± 0.47	1.01 ± 0.47	2.69 ± 0.22	54.70 ± 0.09	1.09 ± 0.03
Leaves	1.67 ± 0.31	1.64 ± 0.47	10.70 ± 2.30	4.04 ± 0.47	0.60 ± 0.47	-	30.57 ± 0.12	-

[a] Mass sugar units/mass dry extractive-free wood. [b] Sample was analyzed extractive-free.

4.3.3 Cellulose Analysis

The CP/MAS ^{13}C NMR spectrum and cellulose signal assignments are presented in Figure 30. The region in the NMR spectrum of cellulose between δ 80 and 92 is the most informative region, which is assigned to C-4 carbons [64]. The area between δ 86 and 92 represents the crystalline and *para*-crystalline forms of cellulose, whereas the broader signal between δ 80 and 86 corresponds to the amorphous domains [64].

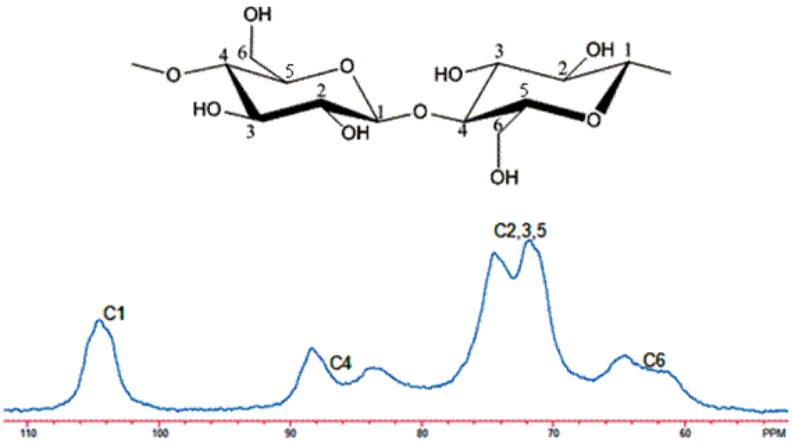


Figure 30. Solid-state CP/MAS ^{13}C NMR spectrum of native *Buddleja davidii* cellulose.

In order to determine the relative amounts of cellulose I_α, cellulose I_β, *para*-crystalline cellulose, and celluloses at accessible and inaccessible surfaces, an analysis method based on a non-linear least-square fitting of CP/MAS ^{13}C NMR spectra was performed [63, 64]. Figure 31 shows the spectral fitting for the C-4 region of the CP/MAS ^{13}C NMR spectrum of cellulose, and the assignments of the signals are presented in Table 34. The cellulose crystallinity index was determined to be 0.55, which is high compared with that of aspen (0.47) and spruce (0.45) [173]. The highly crystalline structure of *B. davidii* cellulose suggests that there may be a need to tailor pretreatment technologies to decrease this value.

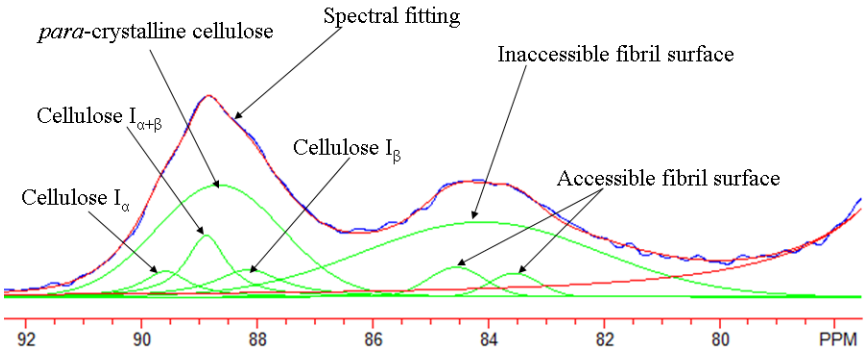


Figure 31. Spectral fitting for the C-4 region of CP/MAS ^{13}C NMR spectrum of native *Buddleja davidii* cellulose.

Table 34. Assignments of signals in the C-4 region of CP/MAS ^{13}C NMR spectrum obtained for native *Buddleja davidii* cellulose.

Assignments	Chemical Shift (ppm)	FWHH[a] (Hz)	Intensity (%)	Line type
Cellulose I_α	89.6	96	4.2	Lorentz
Cellulose $I_{\alpha+\beta}$	88.9	85	8.7	Lorentz
para-crystalline cellulose	88.7	258	32.9	Gauss
Cellulose I_β	88.2	142	6.5	Lorentz
Accessible fibril surface	84.6	116	3.9	Gauss
Inaccessible fibril surface	84.1	482	41.1	Gauss
Accessible fibril surface	83.6	101	2.7	Gauss

[a] FWHH: Full width at half-height.

The values determined from the GPC analysis of cellulose were as follows: the weight-average molecular weight ($\overline{M}_w$) = 5.21×10^5 g/mole, the number-average molecular weight ($\overline{M}_n$) = 3.34×10^4 g/mole, the polydispersity index ($\overline{M}_w / \overline{M}_n$) = 15.6, and the weight-average degree of polymerization (DP_w) = 1004. The DP of cellulose in *B. davidii* is low compared to that of aspen (2500) and spruce (3300) [77]. In contrast with the higher crystallinity index (see above), this lower DP_w may be beneficial in the hydrolysis of cellulose by cellulase enzymes.

4.3.4 Structural Analysis of *B. davidii* MWL Using Quantitative ^{13}C NMR

The MWL yield was 0.56% w/w of dry extractive-free wood. The elemental composition (% w/w dry MWL) was determined to be 60.48% C, 5.73% H, and 32.68% O, which resulted in chemical composition of $C_9H_{10.23}O_{3.65}$. The quantitative ^{13}C NMR spectrum is presented in Figure 32. This technique provides a facile method to determine the amount of various functional groups and lignin structures. A small peak of cinnamaldehyde was observed in the chemical shift of 194 ppm, which was also

confirmed in the DEPT-135 spectrum. The region between δ 160 and 103 corresponds to the six aromatic carbons plus four vinyl carbons; two from side chain of cinnamaldehyde unit and two from side chain of cinnamyl alcohol unit. The integral value from δ 160 – 103 minus the integral values of the vinyl carbons of both cinnamaldehyde and cinnamyl alcohol is set as the reference for quantifying the lignin structures as well as the functional groups. So the integral values for the structural moieties are reported per aryl group [174]. Table 35 summarizes the various lignin moieties, the chemical shifts, and the number/aryl group. The spectrum regions for *p*-hydroxyphenyl (*h*) units, aromatic quaternary C, and aromatic tertiary C have δ-range of 162-157, 160-123, and 123-103 respectively. The DEPT-135 ^{13}C NMR spectrum confirms the chemical shifts for the CH_3, CH_2, and CH regions (Figure 33). No *p*-hydroxyphenyl lignin was detected by NMR. The guaiacyl-to-syringyl ratio (*g:s*) ratio was calculated based on number of carbons per aromatic ring in C-2 of guaiacyl unit and C-2/C-6 of syringyl unit [48]. Since an uncondensed syringyl unit has two tertiary carbons (i.e. C-2/C-6), an approximate content of syringyl moieties/aryl group in the *B. davidii* MWL can be estimated from the total number of C-2/C-6 carbons divided by 2 (Table 35), i.e. 0.18/aryl group ($I_{108\text{-}103}/2$). Because *h*-units in the MWL are negligible as indicated by the quantitative ^{13}C NMR, the MWL consists of *g* and *s* with an approximate ratio of 81:19 (*g:s*). This ratio is low compared to other hardwood species such as: birch (50:50) or *Eucalyptus grandis* (36:62) [48].

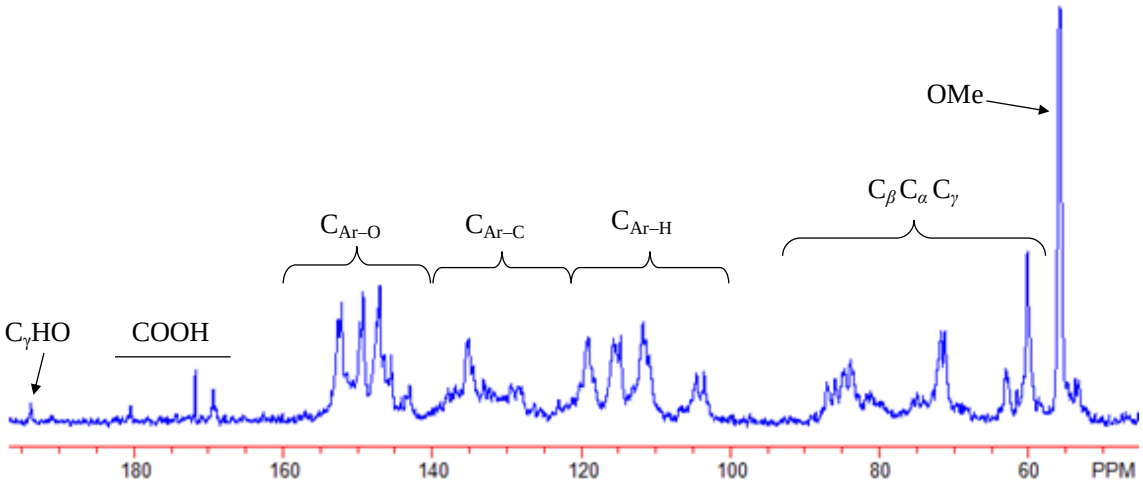


Figure 32. Quantitative ^{13}C NMR spectrum of milled wood lignin from native *Buddleja davidii*.

Table 35. Quantification of several lignin moieties in *Buddleja davidii* milled wood lignin using ^{13}C NMR.

Structure	δ ^{13}C–NMR (ppm)	Number/Aryl group
γ-C=O in cinnamaldehyde	194.9 – 193.0	0.03
Unconjugated carboxylic C=O	172.8 – 171.2	0.04
Unconjugated carboxylic C=O	170.7 – 168.8	0.07
Aromatic C–O bond: ***s***-3,5[a]; ***g***-3,4[b]	160.0 – 140.0	2.20
Aromatic C–O bond: ***s***-4; ***g***-4, and Aromatic C–C bond: C1	140.0 – 123.0	1.44
Aromatic C–H bond: ***g***-6	123.0 – 117.0	0.70
Aromatic C–H bond: ***g***-5	117.0 – 114.0	0.54
Aromatic C–H bond: ***g***-2	114.0 – 108.0	0.76
Aromatic C-H: ***s***-2,6	108.0 – 103.0	0.36
C_β in β–O–4; C_α in β–5 and β–β	90.0 – 78.0	0.97
C_α in β–O–4	78.0 – 67.0	0.93
Cγ in β–5, β–O-4 with α-C=O, and cinnamyl alcohol	65.0 – 61.3	0.25
Cγ in β–O–4 without α-C=O	61.3 – 58.0	0.56
Methoxyl OCH_3	58.0 – 54.0	1.17
C_β in β–β and C_β in β–5	54.0 – 52.0	0.17

[a] Carbon 3 and 5 in syringyl lignin. [b] Carbon 3 and 4 in guaiacyl lignin .

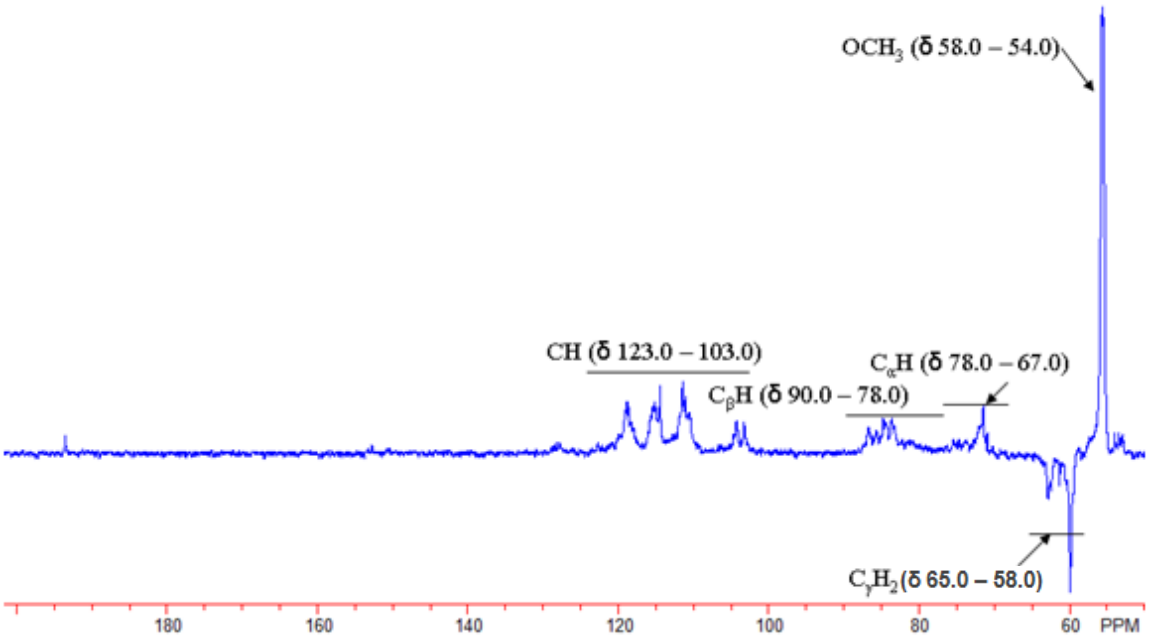


Figure 33. DEPT-edited ^{13}C NMR spectrum of milled wood lignin from native *Buddleja davidii*.

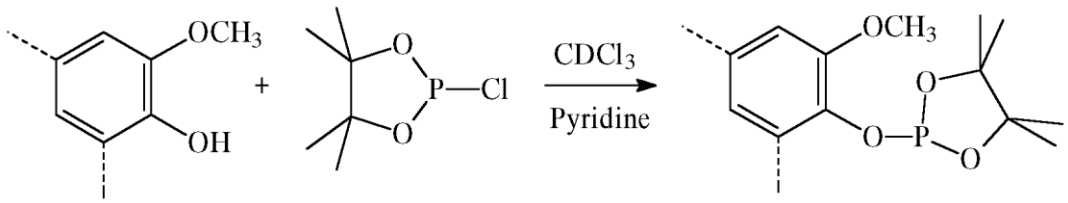
The degree of condensation (DC) in lignin is 45%, which is higher than that of Loblolly Pine (40%) [65] and *Eucalyptus grandis* (21%) [48]. According to Capanema *et al*., the degree of condensation can be determined by subtracting the experimental value of $C_{Ar–H}$ from the theoretical value of $C_{Ar–H}$ [48]. The experimental value is the integral of the tertiary aromatic carbon region (δ 123.0 – 103.0), which is equal to 2.36 Carbons/Aromatic ring (Table 35), and the theoretical value (2.81 Carbons/Aromatic ring) is calculated from the following equation [48]:

$$\text{Theoretical } C_{Ar–H} = 2\boldsymbol{s} + 3\boldsymbol{g} + 2\boldsymbol{h}$$

4.3.5 Structural Analysis of *B. davidii* MWL Using Quantitative ^{31}P NMR

This technique is based on the derivatization of phenolic structures with 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP) (Equation 7) [162]. The

quantitative ^{31}P NMR spectrum for *B. davidii* MWL is presented in Figure 34. The concentration of each hydroxyl functional group was calculated based on the internal standard and the integrated peak area (Table 36). The dominant OH group in the lignin is at aliphatic sites. The lignin has considerable amount of free guaiacyl OH, but low carboxylic acid OH. *B. davidii* lignin is similar to that of *P. tremuloides* (a hardwood), with high aliphatic OH content but low phenolic and carboxylic OH, compared to some other plant species MWL, such as loblolly pine, black spruce, miscanthus, wheat straw, and switchgrass (Table 37).



Equation 7. Derivatization of phenolic structures with 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP).

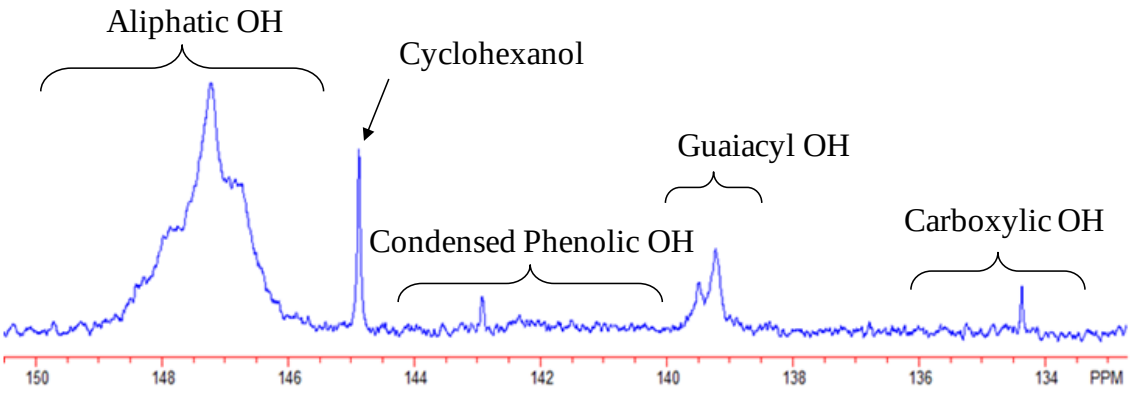


Figure 34. Quantitative ^{31}P NMR of milled wood lignin from native *Buddleja davidii*.

Table 36. Quantification of several hydroxyl functional groups in *Buddleja davidii* milled wood lignin using ^{31}P NMR.

Structure	δ ^{31}P–NMR (ppm)	Concentration (mmol/g lignin)
Aliphatic OH	150.0 – 145.5	4.51
Condensed phenolic OH	144.7 – 140.2	0.27
Guaiacyl	140.2 – 139.0	0.43
Carboxylic acid OH	136.6 – 133.6	0.03

Table 37. Comparison between hydroxyl content in *Buddleja davidii* milled wood lignin and other wood species.

Species	Aliphatic OH (mmol/g lignin)	Phenolic OH (mmol/g lignin)	COOH (mmol/g lignin)
B. davidii	4.51	0.70	0.03
Loblolly pine [65]	4.16	0.77	0.02
Black spruce [50]	4.27	1.13	0.21
Wheat straw [175]	3.49	1.46	0.12
Miscanthus [176]	4.00	1.53	0.13
Switchgrass [11]	3.88	1.00	0.29
P. tremuloides [177]	5.72	0.74	0.06

4.3.6 GPC Analysis of *B. davidii* MWL

The values determined from the GPC analysis were as follows: the weight-average molecular weight ($\overline{M}_w$) = 1.68x10^4 g/mole, the number-average molecular weight ($\overline{M}_n$) = 7.26x10^3 g/mole, and the polydispersity index ($\overline{M}_w/\overline{M}_n$) = 2.31. A value of 2.31 for the polydispersity index indicates a relatively large distribution of molecular weights in this lignin sample. *B. davidii* has higher $\overline{M}_w$ and $\overline{M}_n$ compared to other plant species but lower polydispersity index (except than switchgrass) (Table 38).

Table 38. Comparison between the weight-average molecular weight ($\overline{M}_w$), the number-average molecular weight ($\overline{M}_n$), and the polydispersity index (D) ($\overline{M}_w / \overline{M}_n$) of *Buddleja davidii* milled wood lignin and other species [11, 50].

Species	$\overline{M}_w$ (g/mol)	$\overline{M}_n$ (g/mol)	D
B. davidii	16800	7260	2.31
Douglas fir	7400	2500	2.96
White fir	8300	2800	2.96
Redwood	5900	2400	2.46
Southern pine	14900	4700	3.17
E. globulus	6700	2600	2.58
Switchgrass	5000	2940	1.70

4.4 Conclusions

Buddleja davidii has several unique features ranging from its distribution and growth habitat to aspects of its composition (analyzed here for the first time) that make it a potentially interesting bioresource for biofuels. The stem contains 30% lignin, 35% cellulose, and 34% hemicellulose. The principal hemicellulose is xylan and the biomass has 22% xylose content based on oven-dried weight of extractive-free wood. It has a low cellulose DP_w of 1000 compared with 2500 to 3500 for other wood cellulose. The predominant crystalline form of cellulose is *para*-crystalline cellulose (33%). Both guaiacyl and syringyl lignins are found in this plant with ***g:s*** ratio of 81:19.

Although *B. davidii* has several 'positive' features, it has some undesired characteristics such as relatively high lignin and hemicellulose content, low cellulose content, and a high cellulose crystallinity index. Saccharification and fermentation yields and pretreatment evaluations will be needed to determine the balance of these positive and negative factors for assessing the biofuel production potential of *B. davidii* biomass.

CHAPTER 5

EFFECT OF ETHANOL ORGANOSOLV PRETREATMENT ON ENZYMATIC HYDROLYSIS OF *BUDDLEJA DAVIDII* STEM BIOMASS [2]

5.1 Introduction

Lignocellulosic materials are potential resources for the production of bioethanol. The biochemical process of converting biomass to bioethanol consists of three main steps: pretreatment, enzymatic hydrolysis, and fermentation [7]. In this bioconversion route, pretreatment is probably the most crucial step as it has a large impact on the efficiency of the overall process [7, 10]. The applied pretreatment conditions will have a significant effect on the characteristics of the substrate, which in turn dictates the effectiveness of the enzymatic hydrolysis by cellulases, which hydrolyze the β-1,4-glucosidic linkages of cellulose [10, 178]. Useful pretreatment approaches need to have the ability to alter lignin and hydrolyze the hemicelluloses so that cellulose can become more accessible to the action of the cellulase enzymes [111]. Without pretreatment, enzymatic hydrolysis is ineffective because the hemicellulose and lignin matrix obstructs

[2] This manuscript was accepted for publication in Industrial & Engineering Chemistry Research, 2010. It is entitled as "Effect of Ethanol Organosolv Pretreatment on Enzymatic Hydrolysis of *Buddleja davidii* Stem Biomass". The other authors are Poulomi Sannigrahi, Yunqiao Pu, and Arthur J. Ragauskas from the Institute of Paper Science and Technology and School of Chemistry and Biochemistry at Georgia Institute of Technology and Michael Ray and Richard J. Murphy, from the Division of Biology at Imperial College London, UK.

the cellulases from hydrolyzing the native cellulose into monomeric glucose, appropriate for fermentation to ethanol [7].

The ethanol organosolv pretreatment [2, 129, 130, 132, 134, 179] is one of the various pretreatment technologies that are being studied besides alkali [112, 113], dilute-acid pretreatment [122, 128, 180, 181], and SO_2-catalyzed steam pretreatment [182]. Organosolv pretreatment is of interest because it has several advantages: (1) organic solvents are easy to recover by distillation and recycled for pretreatment; (2) the recovered lignin after pretreatment has desirable characteristics that can be used for several coproducts; (3) the pretreatment considers the utilization of all the biomass components, which makes it feasible for biorefining of lignocellulosic biomass [130, 131]. Consequently, the organosolv technology is now being developed as part of a commercial lignocellulose biorefinery, known as Lignol [130].

In an organosolv pretreatment, the biomass is treated in an aqueous-organic solvent mixture with the addition of an acid catalyst, such as H_2SO_4 [7, 129, 132]. Typically, solvents such as methanol, ethanol, acetone, ethylene glycol, triethylene glycol, and phenol are used in organosolv processes [7]. After pretreatment, the hydrolyzed lignin dissolves in the organophilic phase and is recovered as a filtrate via a precipitation reaction (ethanol organosolv lignin (EOL) fraction), the cellulose is recovered in the solid residue (solid fraction), and the hemicellulose is recovered in the water-soluble fraction as monomeric/oligomeric sugars [7, 129]. The cellulose-rich solid fraction can then be enzymatically hydrolyzed using cellulase and β-glucosidase enzymes [134]. Pan and co-workers demonstrated the effectiveness of the organosolv pretreatment on both hardwood [129] and softwood [132]. High enzymatic conversion of cellulose to

glucose, in the range of 90-100%, was obtained because most of the lignin and hemicelluloses were hydrolyzed and removed from the solid substrate, and the "purified" cellulose was amenable to the enzymatic deconstruction.

Enzymatic hydrolysis of cellulose requires several cellulases: endoglucanases (endo-1,4-β-glucanases, EG) (EC 3.2.1.4), cellobiohydrolases (exo-1,4-β-glucanases, CBH) (EC 3.2.1.91), and cellobiases (β-glucosidases) (EC 3.2.1.21) [183, 184]. EG cleaves internal glycosidic bonds, while CBH acts on glucan chain ends, releasing cellobiose molecules, which are then hydrolyzed to glucose by cellobiase [85, 183, 184]. CBHs are capable of degrading both crystalline and amorphous cellulose efficiently [178]. These enzymes contain catalytic domains and carbohydrate-binding molecules (CBMs) that act together to hydrolyze cellulose [185]. The CBM binds to the cellulose surface and decrystallizes the glucan chain, thus forcing it towards the active-site tunnel of the catalytic domain and hydrolyzing the cellulose microfibrils to its monomeric unit through either the inversion or retention mechanism [16, 143, 178].

Many researchers have focused on the relationship between the characteristics of pretreated biomass and its enzymatic hydrolyzability [10, 85, 134, 143]. The general factors that affect enzymatic digestibility of cellulose in biomass are lignin content, hemicelluloses content, crystallinity of cellulose, and degree of polymerization of cellulose. Lignin acts as an inhibitor to cellulose [127], and the other factors as well as lignin impede the cellulase-cellulose interaction [143, 186]. However, the correlation of these properties to the susceptibility of the biomass to enzymatic deconstruction is substrate and pretreatment dependent [143]. Studying the changes in the structure of cellulose during organosolv pretreatment is an area of research that remains to be

thoroughly investigated. Native cellulose in plants is a composite of three different crystalline allomorphs, cellulose I_α, cellulose I_β, and *para*-crystalline cellulose, and two noncrystalline forms, amorphous cellulose at accessible and inaccessible fibril surfaces [61-64]. Cellulose I_α, a one chain triclinic unit cell, is the dominant form in bacterial and algal cellulose, whereas cellulose I_β, a monoclinic two-chain unit cell, is dominant in higher plants, such as wood [64]. *para*-Crystalline cellulose is the form that is less ordered than cellulose I_α and cellulose I_β but more ordered than amorphous cellulose [64]. Accessible fibril surfaces are those in contact with water, while the inaccessible fibril surfaces are fibril-fibril contact surfaces and surfaces resulting from distortions in the fibril interior [65]. Solid-state cross polarization/magic angle spinning (CP/MAS) ^{13}C NMR is a technique that has been used to determine the relative intensities of these various cellulose structures. The technique is based on a line-shape spectral fitting analysis of the C-4 region of the solid-state CP/MAS ^{13}C NMR spectra, where Lorentzian lines are used to fit the I_α and I_β signals, and Gaussian lines are used to fit the signals from the other remaining structures [61-64].

In previous work [187], *Buddleja davidii* was presented as a potential bioresource for bioethanol production. This plant has been used as a source to extract a variety of high-value chemicals, such as anti-inflammatory and antioxidant compounds [31]. As society moves toward the concept of biorefinery, this plant may well be cultivated for value added chemicals, and the plant biomass residue could then be a viable resource for biofuel production. Biomass characterization and the negative and positive features of this plant were reported [187]. Briefly, this perennial plant has a wide range of growth habitat and favorable growth dimensions. It has relatively high lignin (30%) and

hemicellulose (34%) contents and low cellulose content (35%) with a cellulose crystallinity index of 0.55 [187]. To determine the balance between these positive and negative factors on potential glucose yields and hence bioethanol production potential, evaluation of pretreatment and enzymatic hydrolysis behavior of *B. davidii* is needed. This paper presents research on the ethanol organosolv pretreatment of *B. davidii* and its ability to provide an enzymatically hydrolysable substrate. Furthermore, the study explored the fundamental characteristics of pretreated *B. davidii* in the context of developing an efficient bioconversion of cellulose to glucose.

5.2 Experimental Section

5.2.1 Biomass Material Preparation

Buddleja davidii stems were obtained from a single plant from a domestic garden in southern England in March 2008 and prepared as described in Chapter 3 (3.1.2.2 *Buddleja davidii* for Ethanol Organosolv Pretreatment Experiment).

5.2.2 Ethanol Organosolv Pretreatment

The ethanol organosolv process was performed as described in Chapter 3 (3.2.4 Ethanol Organosolv Pretreatment). The organosolv pretreatment was performed at six different conditions shown in section 3.2.4 Ethanol Organosolv Pretreatment.

5.2.3 Analytical Procedures

All compositional analyses were determined on the oven dry weight of samples. The carbohydrate profile and lignin content were measured as described in Chapter 3 (3.3.1 Carbohydrates and Lignin Content). For the water-soluble fraction, the analyses for

soluble lignin and monosaccharides were conducted in the same manner as in the solid fraction, except for the sample preparation. Before autoclaving, the pH of an aliquot (20.00 mL) from the water-soluble fraction was adjusted with 72% sulfuric acid, according to the Laboratory Analytical Procedure (LAP) provided by the National Renewable Energy Laboratory (NREL) [188]. Furfural and HMF contents were determined using the UV absorbance method described by Martinez *et al.* [189]. The typical errors associated with the carbohydrate measurements were ±0.01 to ±2.83, lignin measurements were ±0.08 to ±0.73, and furfural and HMF measurements were ±0.02.

5.2.4 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed as described in Chapter 3 (3.3.3 Enzymatic Hydrolysis).

5.2.5 Cellulose Characterization

Holocellulose (cellulose and hemicellulose) from pretreated wood was first isolated as described in Chapter 3 (3.2.2 Holocellulose Pulping). Then cellulose was extracted as described in Chapter 3 (3.2.3 Isolation of Cellulose, Hemicellulose, and Milled Wood Lignin). Solid-state CP/MAS ^{13}C NMR experiments were performed as described in Chapter 3 (3.3.4.1 Solid-State CP/MAS ^{13}C NMR Characterization of Cellulose).

The number-average molecular weight ($\overline{M}_n$) and the weight-average molecular weight ($\overline{M}_w$) of cellulose were determined by GPC as described in Chapter 3 (3.3.5 Gel Permeation Chromatography Analysis of Cellulose). The number of reducing chain ends of cellulose (F_{RE}) was calculated as 1/DPw [85].

5.3 Results and Discussion

5.3.1 Ethanol Organosolv Pretreatment of *Buddleja davidii*

The composition of the EOL, solid, and water-soluble fractions after the organosolv pretreatment of *B. davidii* at the six conditions are given in Table 39.

Table 39. Compositional analysis of the three fractions after the ethanol organosolv pretreatment of *Buddleja davidii* at various conditions.

	Conditions[b]					
Content[a]	A	B	C	D	E	F
EOL	**4.34**	**8.86**	**18.97**	**7.45**	**11.11**	**8.14**
Solid	**61.95**	**56.07**	**47.05**	**56.81**	**61.12**	**40.48**
Klason lignin	21.14	18.64	9.16	17.90	15.36	12.08
Acid-soluble lignin	0.27	0.38	0.35	0.25	0.44	0.56
Glucose	33.03	32.34	33.46	33.46	33.29	26.22
Xylose	6.10	4.78	3.40	4.38	9.90	1.20
Mannose	1.21	0.65	0.61	0.76	1.67	0.26
Galactose	0.13	0.15	0.05	0.04	0.29	0.11
Arabinose	0.07	0.11	0.02	0.02	0.17	0.05
Water-soluble	**16.20**	**17.30**	**14.90**	**22.78**	**13.07**	**20.68**
Acid-soluble lignin	2.76	3.40	3.56	3.61	3.78	9.58
Glucose	1.12	1.37	1.39	2.84	0.87	3.16
Xylose	9.87	9.00	7.12	13.18	7.13	3.70
Mannose	1.11	1.22	1.12	1.75	0.60	0.69
Galactose	0.64	0.49	0.48	0.69	0.35	0.35
Arabinose	0.36	0.28	0.24	0.32	0.19	0.11
Furfural + HMF	0.34	0.94	0.99	0.39	0.15	3.09

[a] % (w/w) in oven-dried wood. [b]Condition A was carried in 1.25 % w/w H_2SO_4 and 50 % v/v ethanol at 180 °C for 60 min; condition B was carried in 1.75 % w/w H_2SO_4 and 50 % v/v ethanol at 180 °C for 40 min; condition C was carried in 1.50 % w/w H_2SO_4 and 65 % v/v ethanol at 195 °C for 60 min; condition D was carried in 1.25 % w/w H_2SO_4 and 50 % v/v ethanol at 180 °C for 80 min; condition E was carried in 1.50 % w/w H_2SO_4 and 65 % v/v ethanol at 170 °C for 40 min; condition F was carried in 2.00 % w/w H_2SO_4 and 65 % v/v ethanol at 195 °C for 60 min.

The work done by Pan *et al.* on ethanol organosolv pretreatment of poplar [129] was used as a guide for determining the pretreatment conditions for *B. davidii*. Poplar was pretreated under various conditions and the influence of temperature, time, catalyst dose, and ethanol concentration on product yields was analyzed [129]. Thus, the optimum

pretreatment condition found for poplar was used as a starting point for pretreating *B. davidii*. Conditions A, B, and C were further investigated in this study because they produced substrates with different composition and/or enzymatic hydrolysis profiles (Figure 35).

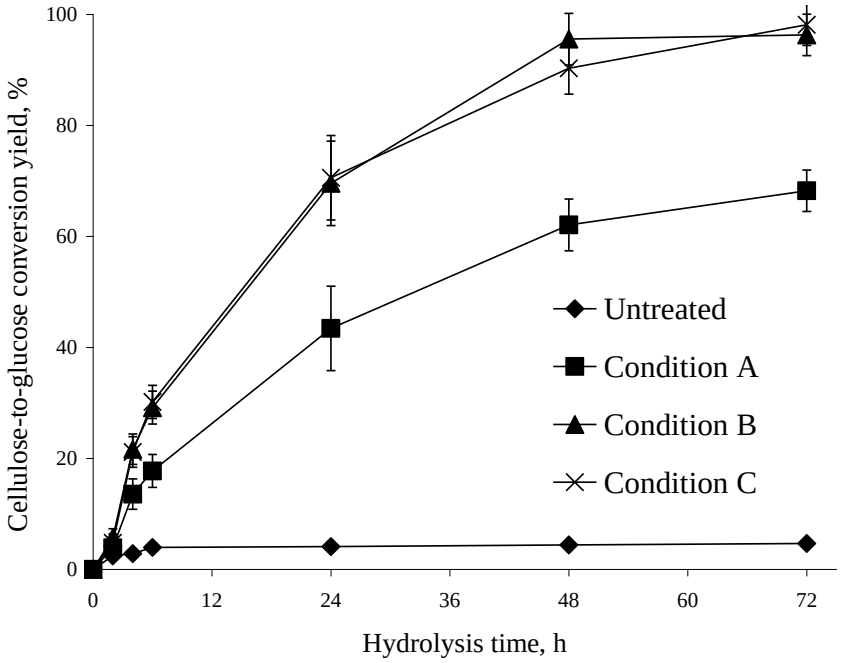


Figure 35. Enzymatic hydrolysis of untreated and ethanol organosolv pretreated *Buddleja davidii*.

As the severity of the pretreatment increased (condition A → condition C), more delignification and carbohydrate hydrolysis/degradation occurred. The increase in delignification was evident by the increase in EOL fraction (~4% to ~19%), decrease in Klason lignin content in the solid fraction (~21% to ~9%), and increase in acid-soluble lignin content in the water-soluble fraction (~2.8% to ~3.6%). The glucose content in the solids for the three conditions was ~33%. Approximately 85% of the original glucose in

the wood was recovered in the solid fraction and about 3% was measured in the water-soluble fraction. This is comparable to the recovery obtained from the organosolv pretreatment of other hardwoods, such as hybrid poplar (86%) [129]. On the other hand, the hemicelluloses were more susceptible to variations in pretreatment conditions. The xylose content in both the solid and water-soluble fractions decreased as the severity of the pretreatment increased. The recovered xylose percentage of the original xylose in wood decreased from ~74% to ~49%, from condition A → C. This could be explained by the increase in furfural and 5-hydroxylmethyl furfural (HMF) contents. Under severe conditions (i.e., high temperature and acidity) sugars degrade to furfural (from pentoses) and HMF (from hexoses) [136].

5.3.2 Enzymatic Hydrolysis of Organosolv Pretreated *Buddleja davidii*

Enzymatic hydrolysis results for the three pretreated and untreated substrates are shown in Figure 35. The significant difference in the percent conversion between untreated and pretreated *B. davidii* emphasizes the importance of pretreatment. Conditions B and C produced substrates that were more readily digestible than substrate A and gave similar hydrolysis profiles. This suggests that while reducing the Klason lignin (KL) content from ~30% (untreated *B. davidii*) to ~19% (substrate from condition B) enhanced the enzymatic hydrolysis, further reduction to ~9% (substrate from condition C) did not confer improved enzymatic hydrolysis. Despite the fact that *B. davidii* has high lignin (~30%) and hemicellulose (~34%) contents, ethanol organosolv pretreatment was able to delignify the wood, hydrolyze the hemicelluloses, and produce readily enzymatically hydrolyzable substrates with high glucose recovery (85% of the original glucose in the wood).

Many studies have shown that lignin removal from the biomass greatly enhances the enzymatic deconstruction of cellulose, since lignin inhibits cellulase activity [123, 127, 145, 186, 190]. In the case of organosolv pretreatment, the relationship between the lignin content in pretreated substrates and their enzymatic digestibility was not found to be proportional.6 Substrates from condition A (~21% KL) and B (~19% KL) exhibited different hydrolyzability, although their lignin contents were essentially the same. Substrate C on the other hand had a substantially lower lignin content (~9% KL) than substrate B (~19% KL), but their hydrolysis profiles were similar. Thus, at least in *B. davidii*, it seems that there are other factors influencing the enzymatic hydrolyzability of biomass in addition to residual lignin content. This lack of correlation between pretreated Klason lignin content of *B. davidii* and enzymatic hydrolysis of cellulose was strongly supported by the fact that even though substrate B still had high lignin content, its enzymatic hydrolysis was very efficient. This characteristic could be advantageous, since low delignification would require less severe conditions, thus minimizing sugar degradation and improving the fermentation process [136].

To explain the above observations, the degree of polymerization (DP) of cellulose extracted from the three substrates was determined. Pan *et al.* showed that substrates with low viscous cellulose were more amenable to enzymatic degradation [132]. Table 40 presents the weight-average molecular weight ($\overline{M}_w$), the number-average molecular weight ($\overline{M}_n$), the polydispersity index ($\overline{M}_w/\overline{M}_n$), and the weight-average degree of polymerization (DPw) of untreated and pretreated cellulose samples. The DPw and the polydispersity index decreased as the severity of the pretreatment increased. Extensive acid-catalyzed depolymerization of cellulose reduced DP and produced a narrower

molecular weight distribution. The similar and low DPw for cellulose B (~500) and C (~400) appear to explain their better hydrolyzability than cellulose A with a DPw of ~900.

Table 40. Gel permeation chromatography (GPC) analysis of untreated and three organosolv pretreated *Buddleja davidii* cellulose samples[a].

	Untreated [187]	Cellulose A	Cellulose B	Cellulose C
$\overline{M}_w$ (g/mole)	$5.21x10^5$	$5.04x10^5$	$2.78x10^5$	$2.18x10^5$
$\overline{M}_n$ (g/mole)	$3.34x10^4$	$3.58x10^4$	$3.51x10^4$	$3.07x10^4$
$\overline{M}_w / \overline{M}_n$	15.6	14.1	7.9	7.1
DP_w	1000	970	530	420
F_{RE} (%)	0.10	0.10	0.19	0.24

[a] Cellulose A, B, and C were isolated from *B. davidii* biomass after pretreatment A, B, and C, respectively (Table 26).

Lower DP improves enzymatic hydrolysis due to two factors: (1) increasing the number of cellulose chain reducing ends and (2) making cellulose more reactive to the enzymes [85]. Ascomycete *Trichoderma reesei* produces CBH I (Cel7A) an enzyme that acts on the reducing chain ends of cellulose [143, 184]. As the DP of cellulose decreased, the number of reducing ends (F_{RE}) of cellulose increased (Table 40). The fraction of reducing ends relative to all glucan units present is basically the reciprocal of DP [85]. Therefore, the higher number of cellulose chain ends in *B. davidii* cellulose pretreated under conditions B and C increased the exoglucanase activity, resulting in a virtually complete hydrolysis of cellulose after 72 h. Furthermore, shorter chains allow cellulose to be more amenable to enzymatic deconstruction because they do not form strong hydrogen bonding (i.e., they form weaker networks permitting greater possibility for enzyme access) [72, 91, 134].

5.3.3 Crystallinity and Ultrastructure of *Buddleja davidii* Cellulose

Solid-state CP/MAS ^{13}C NMR was used to determine both the crystallinity and ultrastructure of cellulose isolated from untreated and pretreated *B. davidii* samples. The spectrum of the cellulose isolated from substrate A and the signal assignments are shown in Figure 36. The C-4 region between δ 80 and 92 is very informative because it corresponds to the crystalline/*para*-crystalline (δ 86-92) and amorphous (δ 80-86) domains [64]. The results of the spectral fitting of C-4 region for cellulose A are presented in Figure 37 and Table 41.

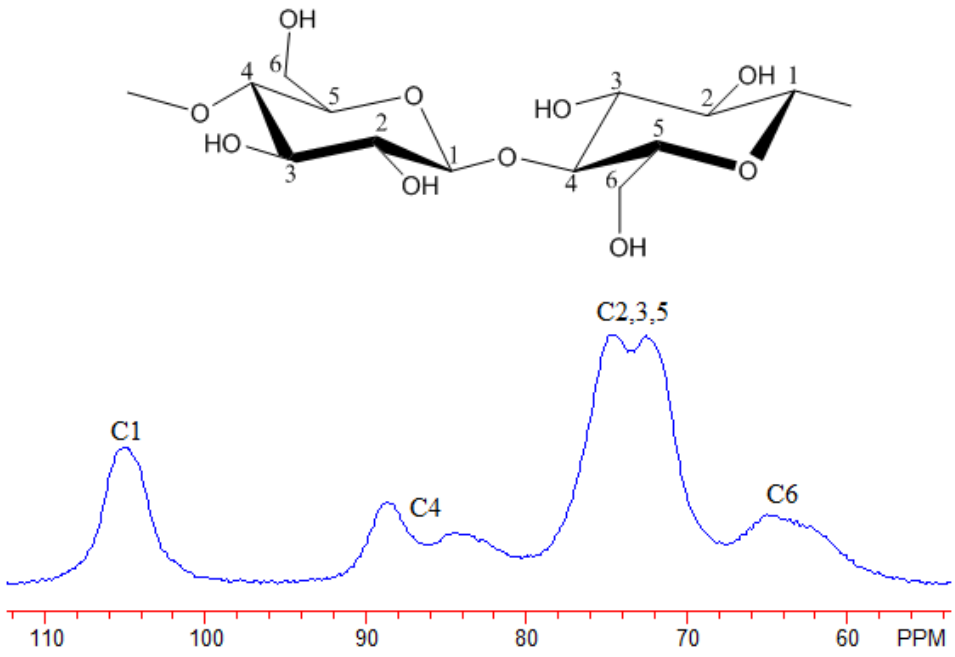


Figure 36. Solid-state CP/MAS ^{13}C NMR spectrum of *Buddleja davidii* cellulose isolated from the substrate prepared at condition A (180°C, 60 min, 1.25% H_2SO_4, and 50% ethanol).

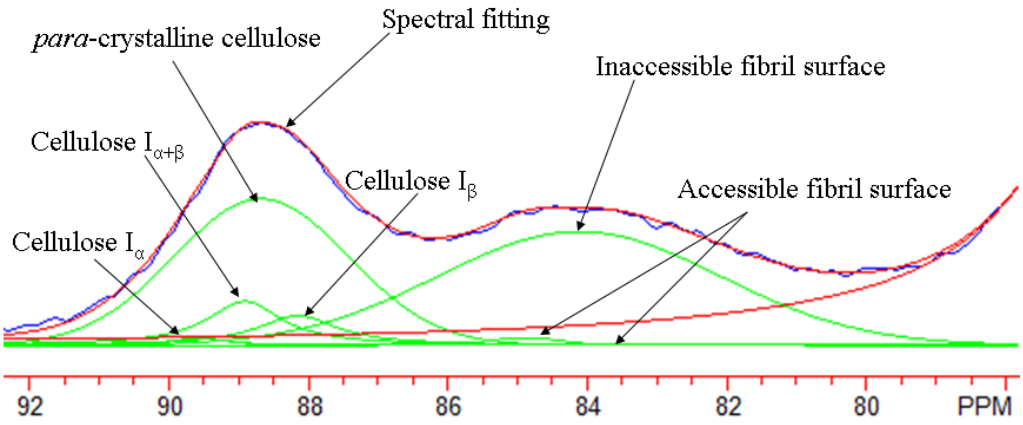


Figure 37. Spectral fitting for the C-4 region of the CP/MAS ^{13}C NMR spectrum of cellulose isolated from substrate A.

Table 41. Results of spectral fitting of the C-4 region of CP/MAS ^{13}C NMR spectrum of cellulose A.

Assignments	Chemical Shift (ppm)	FWHH[a] (Hz)	Intensity (%)	Line type
Cellulose I_{α}	89.6	96	0.9	Lorentz
Cellulose $I_{\alpha+\beta}$	88.9	131	7.4	Lorentz
para-crystalline cellulose	88.7	296	37.8	Gauss
Cellulose I_{β}	88.2	142	5.3	Lorentz
Accessible fibril surface	84.6	116	0.8	Gauss
Inaccessible fibril surface	84.1	480	47.6	Gauss
Accessible fibril surface	83.6	101	0.2	Gauss

[a] FWHH: Full width at half-height.

The crystallinity and ultrastructural features of the various cellulose samples are compared in Table 42. The crystallinity index (CrI) is calculated as the NMR integration value between 92 and 86 ppm divided by the integration value between 92 and 80 ppm **($I_{92\text{-}86}$/ $I_{92\text{-}80}$).**

Table 42. Comparison between the crystallinity and ultrastructure of cellulose isolated from untreated and ethanol organosolv pretreated *Buddleja davidii*.

Assignments	Relative Intensity (%)			
	Untreated Cellulose [187]	Cellulose A	Cellulose B	Cellulose C
Cellulose I_{α}	4.2	0.9	0.9	0.9
Cellulose $I_{\alpha+\beta}$	8.7	7.4	7.2	6.7
para-crystalline cellulose	32.9	37.8	41.0	36.7
Cellulose I_{β}	6.5	5.3	3.7	1.7
Accessible fibril surface at 84.6 ppm	3.9	0.8	0.9	1.1
Inaccessible fibril surface	41.1	47.6	46.2	52.9
Accessible fibril surface at 83.6 ppm	2.7	0.2	0.2	0.0
Crystallinity index	0.55	0.53	0.53	0.49

Cellulose A, B, and C were isolated from *B. davidii* biomass after pretreatment A, B, and C, respectively (Table 26).

The CrI of cellulose A and B showed no change, while cellulose C had a CrI that was 11% less than that of the native cellulose. Thus, as pretreatment severity increased the CrI decreased, indicating that the ethanol organosolv pretreatment is capable of disrupting the crystallinity of cellulose. In contrast, Pan *et al.* reported the changes in crystallinity of cellulose for pretreated Lodgepole pine which increased when the organosolv pretreatment severity increased, suggesting that amorphous cellulose degrades more easily than the more stable crystalline cellulose during pretreatment [134]. Another study showed a decrease in crystallinity of organosolv pretreated pine cellulose [191]. The exact effect of organosolv pretreatment on the crystallinity of cellulose is unknown, and its dependency on biomass resources is not fully defined [131]. These observations could be attributed to the fact that the pretreatment and its effects are dependent on the compositional and structural characteristics of the biomass, as well as

the pretreatment conditions. Additional studies are needed to fully understand how this pretreatment alters the crystallinity of cellulose.

It is generally believed that crystalline cellulose is less amenable to cellulase attack than amorphous cellulose [145]. Jeoh *et al.* observed higher enzymatic accessibility and digestion rates for amorphous cellulose than crystalline cellulose, since there was more extensive binding between *T. reesei* Cel7A and the reducing-ends of amorphous cellulose [143]. In *Buddleja davidii*, the crystallinity of cellulose appears to have had little effect on the enzymatic hydrolysis, possibly because a CrI of 0.55 is already sufficiently low for efficient hydrolysis. Cellulose B had practically the same CrI as the native cellulose and cellulose A, but was easier to digest. Further reduction of CrI to 0.49 in cellulose C afforded no additional benefit as its hydrolysis profile was comparable to that of cellulose B (Figure 35). To thoroughly understand the effects of DP and crystallinity of *B. davidii* cellulose on its enzymatic digestibility, the ultrastructure of the different cellulose samples was determined (Table 42). The dominant structures in untreated *B. davidii* cellulose are *para*-crystalline cellulose (33%) and celluloses at inaccessible surfaces (41%). According to Bergenstråhle *et al.*, the two chemical shift assignments to accessible fibril surfaces (84.6 and 83.6 ppm) arise from the result of glucan chains at pairs of opposite fibril surfaces which rest on different crystallographic planes [192]. After ethanol organosolv pretreatment, the amount of cellulose I_{α} and cellulose I_{β} decreased, whereas the relative intensity of *para*-crystalline and amorphous celluloses increased. From the NMR spectrum (Figure 37), the region between 80 and 86 ppm represents amorphous cellulose, which is illustrated as accessible and inaccessible fibril surfaces. Completely opposite results were observed during the two-step dilute

sulfuric acid pretreatment of Loblolly pine, inferring that the less stable amorphous regions were readily degradable [65]. During organosolv pretreatment, there was decrystallization of cellulose, thereby reducing both crystalline forms I_α and I_β. The concurrent increase in *para*-crystalline cellulose suggests that cellulose I_α and I_β allomorphs are converted to *para*-crystalline cellulose because of the depolymerization of the glucan chains, as indicated by the lower DP. *para*-Crystalline cellulose, with shorter glucan chains, would form weaker networks and have more reducing ends, which are characteristics that allow for more efficient enzymatic hydrolysis.

Pu *et al.* showed that the relative intensities of cellulose I_α, *para*-crystalline cellulose, and the amorphous domains decreased during the enzymatic hydrolysis of fully bleached softwood (pine) kraft pulp, while the relative amount of cellulose I_β increased because it is the more ordered and stable crystal structure (less susceptible to cellulases) [64]. The high relative proportions of *para*-crystalline and amorphous cellulose, and the low percentage of cellulose I_α and I_β in *B. davidii* cellulose B reflect its high conversion to glucose. As the pretreatment severity increased in condition C, the crystalline components decreased along with an increase in the amorphous domains, thus lowering the CrI. This could be due, in part, to the change in *para*-crystalline cellulose. Therefore, since ethanol organosolv pretreatment lowers the DP, reduces the CrI, and increases the relative intensities of the *para*-crystalline and amorphous cellulose structures, it provides *B. davidii* substrates that are readily digestible by cellulases.

5.4 Conclusions

A balance between the attractive features and undesired characteristics of *Buddleja davidii* was determined by performing ethanol organosolv pretreatment followed by enzymatic hydrolysis. The presence of high lignin (30%) and hemicelluloses (34%) contents in *B. davidii* wood was not found to be a negative factor since these biopolymers were easily removed during ethanol organosolv pretreatment. Furthermore, the alteration to the DP, crystallinity index, and the increased relative proportions of more reactive cellulose structures after the pretreatment also acted to yield a readily hydrolyzable material. A total of 85% of the original glucose in the wood was recovered in the solid fraction after the pretreatment, and high cellulose to glucose conversion (~98%) was achieved.

In summary, the following characteristics account for efficient enzymatic deconstruction of *B. davidii* after the ethanol organosolv pretreatment: (1) removal of hemicellulose, (2) delignification, (3) reduction in DP of cellulose, and (4) the conversion of crystalline cellulose dimorphs (I_α/I_β) to the easily degradable *para*-crystalline and amorphous celluloses. Although delignification is an essential factor in enhanced enzymatic saccharification of *B. davidii*, in this study we would assign equal importance to the removal of hemicelluloses and changes in cellulose structure as fundamental characteristics contributing to more efficient enzymatic hydrolysis.

CHAPTER 6

CHEMICAL TRANSFORMATIONS OF *BUDDLEJA DAVIDII* LIGNIN DURING ETHANOL ORGANOSOLV PRETREATMENT[3]

6.1 Introduction

With the emerging necessity to develop alternative sustainable transportation fuels, biofuels have taken a lead position as a viable option to petroleum-derived fuels [8, 20]. The production of cellulosic ethanol, as a potential biofuel, has become the focus of many researchers over the past decade [2, 7]. Lignocellulosic biomass constitutes three biopolymers: cellulose, hemicellulose, and lignin, which together form a complex and rigid structure [1, 12]. This structure causes the biomass to be recalcitrant to biological and chemical degradation [12]. Thus, the biological production of cellulosic ethanol involves three steps: pretreatment, enzymatic hydrolysis, and fermentation. The pretreatment stage is required to reduce the biomass recalcitrance and unlock the polysaccharides, so that they can become accessible to enzymatic hydrolysis [8, 12].

The pretreatment stage is generally considered to be the most costly process and has a significant impact on the efficiency of enzymatic hydrolysis and subsequent fermentation [8-10]. For this reason, researchers have focused on optimizing pretreatment

[3] This manuscript was accepted for publication in Energy & Fuels, 2010. It is entitled as "Chemical Transformations of *Buddleja davidii* Lignin during Ethanol Organosolv Pretreatment". The other authors are Yunqiao Pu and Arthur J. Ragauskas from the Institute of Paper Science and Technology and School of Chemistry and Biochemistry at Georgia Institute of Technology and Michael Ray and Richard J. Murphy, from the Division of Biology at Imperial College London, UK.

conditions as well as understanding the fundamental characteristics of the biomass after pretreatment [10]. Understanding the basic chemical mechanisms involved in pretreatment can be employed to tailor the next generation of more efficient and selective technologies for the viable production of second-generation bioethanol [8]. Another way to improve the overall effectiveness of bioethanol production is to consider the biorefinery scenario, in which all components of biomass are fully used to make a range of fuels, chemicals, materials, heat, and power [1-3]. From this perspective, it is also important to understand how the structure of lignin changes during pretreatment to identify future applications for this valuable bioresource.

Lignin is an amorphous, cross-linked, and three dimensional phenolic polymer that can be biosynthesized from three monolignols: coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol [2]. The exact monolignols employed are plant species dependent and they undergo an *in situ* radical polymerization, yielding several interunit linkages including *β*-*O*-aryl ether (*β*-*O*-4'), resinol (*β*-*β'*), phenylcoumaran (*β*-5'), biphenyl (5-5'), and 1,2-diaryl propane (*β*-1') (Figure 12) [2, 46]. Typical proportions of these linkages in softwood lignin are: 50% *β*-*O*-4', 2% *β*-*β'*, 9-12% *β*-5', 15-25% 5-5', and 7% *β*-1' [2]. Lignin is considered to be the most recalcitrant biopolymer in the plant cell wall, providing structural support, impermeability, and resistance against microbial attack [20].

One of the promising pretreatment technologies for second generation ethanol is ethanol organosolv pretreatment (EOP) because it can convert the recalcitrant lignocellulosic matrix to a reactive cellulosic substrate that can be readily digested by cellulases, resulting in high conversion of cellulose to glucose (90-100%) [129, 132, 134]. The efficient conversion was in part dependent upon the amount of residual lignin

after EOP [130, 132]. Another advantage of EOP is the isolation of sulfur-free lignin that can be used for several co-products [12, 130, 135]. Recently, the conversion of ethanol organosolv lignin (EOL) to a potential fuel precursor for green gasoline/diesel via catalytic hydrogenolysis has been demonstrated [166]. Furthermore, Gellerstedt *et al.* showed the possibility of converting technical lignins to bio-oil by pyrolysis in the presence of formic acid and alcohol [193]. Therefore, with all of the potential applications of lignin, ranging from green fuels to materials and chemicals, understanding its fundamental chemical characteristics is of significant importance.

Recently, we have reported that *Buddleja davidii* can be viewed as an attractive bioresource for bioethanol production because it possesses several attractive agro-energy features [187]. This plant has not yet been cultivated. However, high-value chemicals, such as anti-inflammatory and antioxidant compounds, have been extracted from *B. davidii* [187]. With the concept of biorefinery, this plant may well be cultivated for value-added chemicals, which could subsequently allow for the use of the residual plant biomass as a viable resource for biofuel production. A subsequent publication demonstrated the ability of EOP to produce readily enzymatically hydrolyzable *B. davidii* substrates, providing high (~98%) cellulose-glucose conversion, while the untreated native biomass showed no conversion [194]. The effects of EOP on the characteristics of the pretreated materials were studied, specifically those of cellulose [194]. We found that cellulose chains were extensively depolymerized, and the stable crystalline cellulose dimorphs (I_α/I_β) were converted to *para*-crystalline and amorphous celluloses [194]. This study provides a detailed elucidation of the chemical structure of EOL of *B. davidii* by nuclear magnetic resonance (NMR) and compares the data to that of the native

(untreated) lignin. Four NMR techniques were used to analyze lignin structure: quantitative ^{13}C [48], quantitative ^{31}P [162], qualitative ^{13}C-^{1}H 2D heteronuclear single quantum coherence (HSQC) [174], and distortionless enhancement by polarization transfer (DEPT) [195]. A gel-permeation chromatography (GPC) technique was also used to evaluate the extent of depolymerization of lignin after EOP.

6.2 Experimental Section

6.2.1 Biomass Material Preparation

B. davidii stems were obtained from multiple plants from the Georgia Institute of Technology, Atlanta, GA, in September 2008 and prepared as described in Chapter 3 (3.1.2.2 *Buddleja davidii* for Ethanol Organosolv Pretreatment Experiment).

6.2.2 Ethanol Organosolv Pretreatment

EOP was performed as described in Chapter 3 (3.2.4 Ethanol Organosolv Pretreatment). Three pretreatment conditions were performed as follows: condition 1 was carried out in 1.25% (w/w) H_2SO_4 and 50% (v/v) ethanol at 180 °C for 60 min; condition 2 was carried out in 1.75% (w/w) H_2SO_4 and 50% (v/v) ethanol at 180 °C for 40 min; and condition 3 was carried out in 1.50% (w/w) H_2SO_4 and 65% (v/v) ethanol at 195 °C for 60 min. The produced EOL was recovered by centrifugation, allowed to dry, and purified by Soxhlet extraction with pentane for further analysis.

6.2.3 Structural Analysis of Ethanol Organosolv Lignin

The elemental analysis of EOL (i.e. C, H, and O) was accomplished by Atlantic Microlab, Inc., Norcross, GA. NMR experiments were performed as described in Chapter 3 (3.3.4 NMR Spectroscopy) [11, 158, 162, 163, 174, 187, 196, 197]. NMR data were processed using the TopSpin 2.1 software (Bruker BioSpin). The standard errors associated with ^{13}C and ^{31}P NMR analysis are 3.0% and 1.2%, respectively.

6.2.4 Gel Permeation Chromatography Analysis of EOL

The number-average molecular weight ($\overline{M}_n$) and the weight-average molecular weight ($\overline{M}_w$) were determined as described in Chapter 3 (3.3.7 Gel Permeation Chromatography Analysis of EOL).

6.3 Results and Discussion

6.3.1 Delignification Response

B. davidii biomass was ethanol organosolv pretreated under three conditions as mentioned in the Experimental Section. These conditions were chosen because they exhibited different delignification efficiencies, as summarized in Figure 38 [194]. *B. davidii* has an initial lignin content of ~30% (w/w, oven-dried basis) [187]. Conditions 1, 2, and 3 removed 15, 30, and 64% lignin from the starting material, respectively, indicating that pretreatment 3 was the most severe.

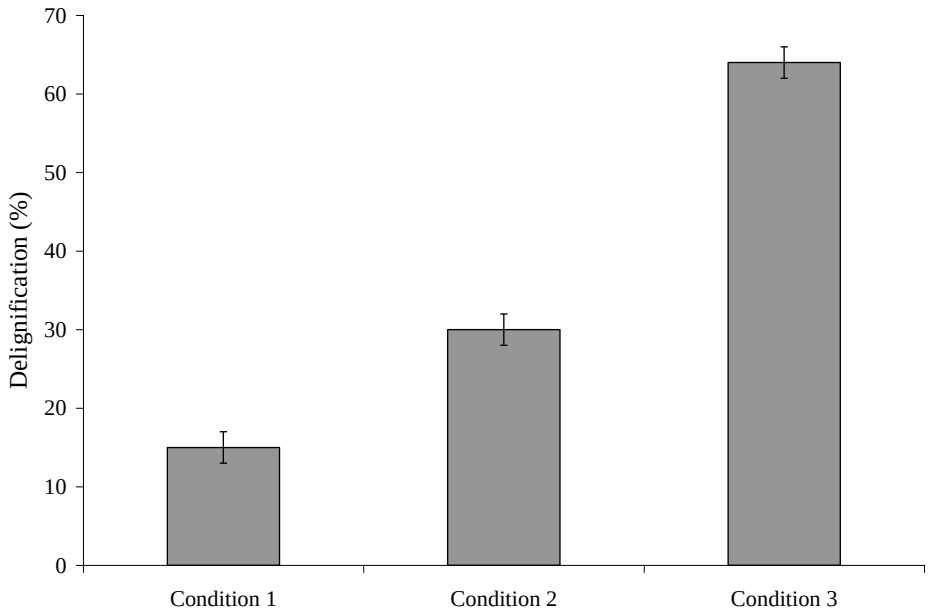


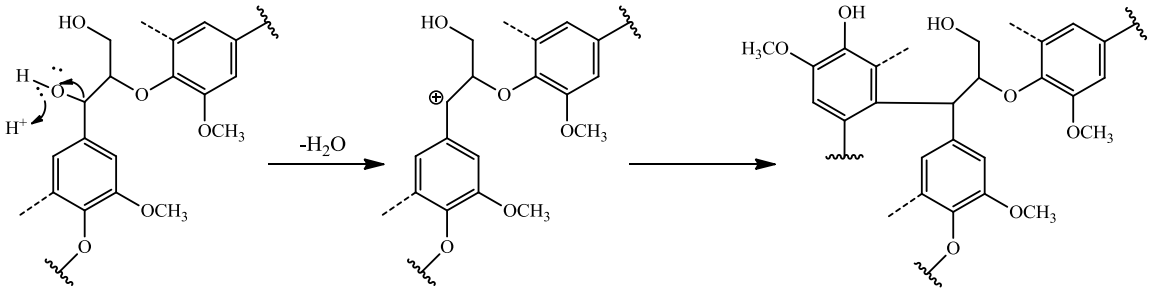
Figure 38. Extent of ethanol organosolv delignification at various pretreatment conditions.

6.3.2 Elemental and Chemical Composition of EOL

The elemental composition of the isolated EOL from pretreatment conditions 1, 2, 3 and the starting milled wood lignin (MWL) are summarized in Table 43. After pretreatment, the carbon content increased while the oxygen content decreased with higher extents of delignification, suggesting that EOL is significantly condensed. Such behavior has been observed in kraft and organosolv lignins [176, 198-200]. A possible reaction route for condensation is described in Equation 8 [201]. Such condensation reactions result from the formation of the carbon cation, normally located at the C_α of the side chain, which can then readily bind with an electron-rich carbon atom in the aromatic ring of another lignin unit (i.e. through the free C_5 or C_6 position) [201, 202].

Table 43. Elemental analysis and chemical composition of MWL and EOL of *Buddleja davidii*.

Sample	Element (% w/w dry lignin)			Chemical Composition
	C	H	O	
MWL[18]	60.48	5.73	32.68	$C_9H_{10.23}O_{3.65}$
EOL 1	63.71	6.04	29.04	$C_9H_{10.26}O_{3.08}$
EOL 2	63.31	5.99	29.63	$C_9H_{10.23}O_{3.16}$
EOL 3	65.11	5.89	28.12	$C_9H_{9.78}O_{2.92}$



Equation 8. An example of lignin condensation reaction during acid-catalyzed treatment of biomass.

6.3.3 Gel-Permeation Chromatography (GPC) Analysis of *B. davidii* EOL

To understand the effect of ethanol organosolv pretreatment on the degree of polymerization (DP) of lignin, GPC analysis was performed to determine the weight-average molecular weight ($\overline{M}_w$) and the number-average molecular weight ($\overline{M}_n$) of EOL. Table 44 summarizes the average molar mass data and the polydispersity index ($\overline{M}_w/\overline{M}_n$) of MWL and EOL. The results clearly indicate that ethanol organosolv pretreatment significantly alters the molecular weight of lignin. The untreated lignin (MWL) had $\overline{M}_w$ of 16800 g/mole, which then decreased by ~85% to 2300–2700 g/mol after pretreatment, suggesting that some interlinkages of the dissolved lignin were

cleaved facilitating lignin depolymerization and solubilization. The estimated DP of lignin decreased from ~80 monomeric units to ~12 monomeric units.

Table 44. Weight-average molecular weight ($\overline{M}_w$), number-average molecular weight ($\overline{M}_n$), and polydispersity index ($\overline{M}_w / \overline{M}_n$) of MWL and EOL.

	$\overline{M}_w$ (g/mol)	$\overline{M}_n$ (g/mol)	$\overline{M}_w / \overline{M}_n$
MWL [187]	16800	7260	2.31
EOL 1	2740	578	4.74
EOL 2	2350	661	3.56
EOL 3	2490	645	3.86

The different pretreatments showed similar $\overline{M}_w$ of EOL, implying that the extent of delignification is not a factor of lignin DP. A 15% delignification (condition 1) gave an EOL of ~13 monomeric units; a 30% delignification (condition 2) gave an EOL of ~11 monomeric units; a 64% delignification (condition 3) gave an EOL of ~12 monomeric units. Based on the three conditions employed, the $\overline{M}_w$ reached an average value of ~2500 g/mol and did not further decrease. One explanation of this observation could be that the EOP, regardless of its severity, attacks certain lignin interlinkages allowing some fractions to become soluble in ethanol. As the severity increased, more of the same interlinkages undergo acid hydrolysis permitting a greater amount of degraded lignin fractions to solubilize in ethanol. These fractions with a DP of ~12 units could contain primarily the types of interlinkages that are recalcitrant to chemical cleavage and would not further degrade. On the other hand, the distribution of the molecular weights became broader after pretreatment as indicated by the higher polydispersity indexes of EOL compared to MWL. The increase in the polydispersity index is due to the simultaneous competition between degradation (depolymerization) and condensation

(repolymerization), which causes an increase in the heterogeneity of the resulting lignin material [201]. To thoroughly understand these MW results, a set of NMR experiments were performed.

6.3.3 Structural Analysis of *B. davidii* EOL Using Quantitative ^{13}C, DEPT-135 and 2D NMR

^{13}C NMR is a technique that is frequently used to determine the amount of several lignin interlinkages, providing a comprehensive overview of the structure of the lignin macromolecule. Quantitative ^{13}C NMR spectra of MWL [187] and EOL 3 are presented in Figure 39. Table 45 compares the quantitative ^{13}C NMR data of the four lignin samples.

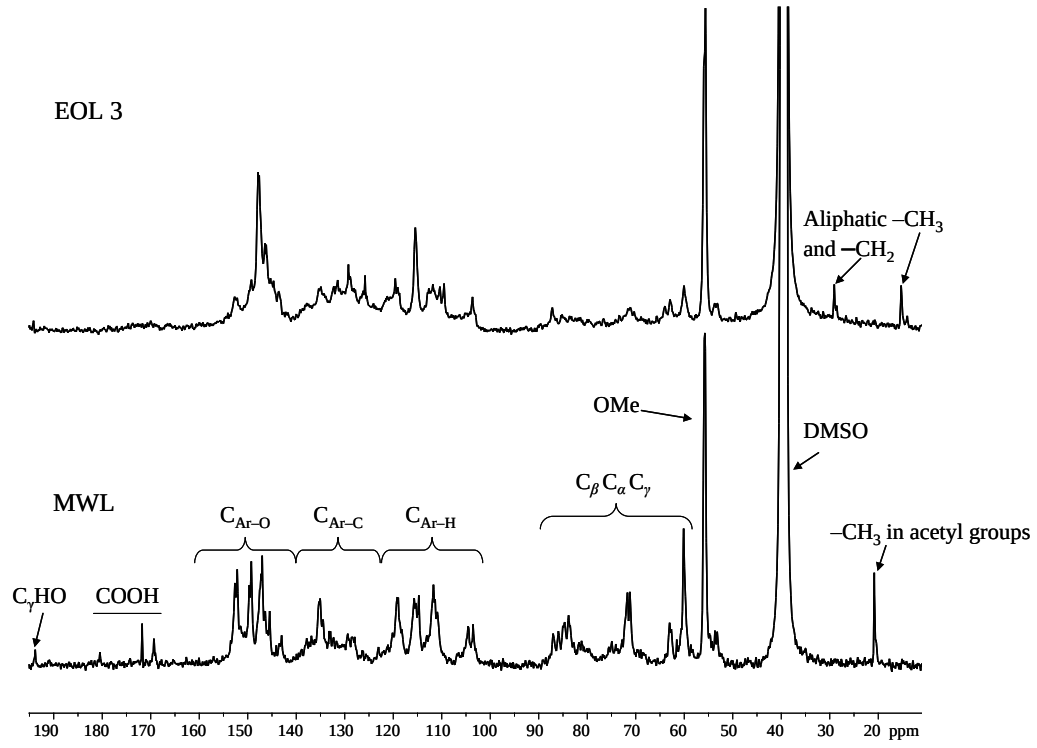


Figure 39. Quantitative ^{13}C NMR spectra of *B. davidii* MWL and EOL 3.

Table 45. Quantitative comparison between MWL and EOL based on the ^{13}C NMR spectra.

	MWL [187]	EOL 1	EOL 2	EOL 3
s/g	0.23	0.25	0.23	0.22
Degree of condensation (%)	45	68	76	82
Methoxyl content (#/aryl group) (δ 58.0–54.0)	1.17	1.17	1.05	1.00
β-O-4′ groups (#/aryl group) (δ 61.3–58.0)	0.56	0.36	0.30	0.24
C_β in *β-β′* & *β*-5′ (#/aryl group) (δ 54.0–52.0)	0.17	0.19	0.12	0.13
Aromatic C–O (#/aryl group) (δ 160.0–140.0)	2.20	2.13	2.15	2.16
Aromatic C–C (#/aryl group) (δ 140.0–123.0)	1.44	1.73	1.80	1.84
Aromatic C–H (#/aryl group) (δ 123.0–103.0)	2.36	2.14	2.05	2.00

The absence of signals between δ 102–90 ppm in the ^{13}C spectrum of EOL 3 (Figure 39) indicates very low carbohydrate content, suggesting that lignin-carbohydrate complex (LCC) structures were hydrolyzed [7, 203]. The integral value of the ^{13}C NMR spectral region δ 160–103 ppm corresponds to the six aromatic carbons after subtracting the four vinyl carbons of cinnamaldehyde unit (δ 194 ppm) and cinnamyl alcohol unit (δ 61.7 ppm) [187]. It is set as the reference for quantifying the lignin structures as well as the functional groups [187]. Thus, the various structural moieties of lignin are reported per aryl group (Ar) (Table 45). The spectrum regions for *p*-hydroxyphenyl (***h***) units, aromatic quaternary C, and aromatic tertiary C are δ 162–157 ppm, δ 160–123 ppm, and δ 123–103 ppm, respectively [187]. No ***h*** units were detected by NMR. Therefore, the syringyl-to-guaiacyl ratio (***s/g***) was calculated based on number of carbons per aromatic ring in C_2 of guaiacyl and C_2/C_6 of syringyl units [48, 187]. The integral value between δ 108–103 ppm divided by 2 can then estimate the content of syringyl moieties, and the guaiacyl moieties were determined by the integral value between δ 114–108 ppm.

The results in Table 45 indicate that the ***s/g*** remained relatively constant after pretreatment. However, it is noteworthy to mention that both ***s*** and ***g*** lignin content

decreased after the most sever pretreatment condition 3. MWL has 0.18 ***s*** units/Ar and 0.76 ***g*** units/Ar [187], while EOL 3 has 0.14 ***s*** units/Ar and 0.63 ***g*** units/Ar. The decrease is due to condensation reactions occurring on C_2, C_5 or C_6 of the aromatic ring of lignin resulting in the formation of new structures that shift these signals downfield. On the other hand, a study on the structural changes of *Miscanthus x giganteus* during different organosolv processes showed that the ***s***/***g*** of the lignins increased after the treatment, implying that condensation took place preferably at ***g*** units [203]. In the case of *B. davidii*, both guaiacyl and syringyl lignin were reactive towards condensation, since the ratio did not change. The methoxyl content decreased by ~11% after pretreatment 2 and 3. EOL isolated from pretreatment condition 1 showed no demethylation of lignin with $-OCH_3$ content of 1.17/Ar equal to that of MWL.

The degree of condensation (DC) of MWL, EOL 1, EOL 2, and EOL 3 were 45% [187], 66%, 76%, and 82%, respectively, and progressively increased with higher degree of delignification. These results are in agreement with the elemental analysis data. The DC values were determined by subtracting the observed signal intensity for $C_{Ar–H}$ (δ 123–103) from the theoretical value of $C_{Ar–H}$ [48]. Lignin condensation is a counterproductive process since lignin must be depolymerized to be removed from the cell wall. However, although condensation reactions occurred during EOP, the MW of EOL did not increase nor was the delignification efficiency impeded by the highly condensed nature of EOL.

In general, the literature suggests that aryl-*O*-aliphatic linkages, such as *β*-*O*-4' are more susceptible to hydrolysis during ethanol organosolv pretreatment [202]. The data in Table 45 clearly shows that the amount of *β*-*O*-4' in the dissolved EOL samples decreased. After pretreatment 1, 2, and 3, the content of *β*-*O*-4' decreased by 36%, 46%,

and 57%, respectively. Other interlinkages such as *β*-5' and *β*-*β′* can be estimated as a sum from the ^{13}C NMR spectral region between 54.0–52.0 ppm (Table 45). This data indicates that some condensation occurred at C_β position after pretreatment 1 making more *β*-5' or *β*-*β′* like units, but as more delignification accompanied pretreatment 2 and 3, a slight degradation of these units occurred. Although more *β*-*O*-4' linkages were cleaved as the pretreatment severity increased, the MW of lignin did not change between EOL 1, 2, and 3. This suggests that the increase in the extent of *β*-aryl ether bond breakage allow more condensation reactions to occur. After pretreatment 3, the content of *β*-*O*-4' units was 57% less than the original content in MWL, while the DC was about double the initial degree in MWL.

The aromatic region in the ^{13}C NMR spectrum (δ 160.0–103.0) can be divided into three categories: oxygenated aromatic carbons (δ 160.0–140.0), aromatic carbon–carbon structures (δ 140.0–123.0), and aromatic methine carbons (δ 123.0–103.0) [158, 174]. After pretreatment, the amount of oxygenated carbons per aryl group did not significantly change (Table 45). The oxygenated aromatic carbons include C_3, C_4, and C_5. On the other hand, the content of aromatic carbon–carbon structures increased by 20%, 25%, and 28% after pretreatment 1, 2, and 3, respectively. This is attributed to the formation of more C–C lignin linkages, such as the one shown in Equation 8. This is also supported by the decrease in number of aromatic methine carbons, which include C_2, C_6, and uncondensed C_5. With increasing DC values, the $C_{Ar–H}$ content gradually decreased by 9%, 13%, and 15% for EOL 1, 2, and 3, respectively. From the chemical composition data in Table 43, the decrease in the hydrogen content per C_9 (from 10.23/C_9 to 9.78/C_9) agrees with the NMR results.

To further understand the structural changes in *B. davidii* lignin after ethanol organosolv pretreatment, the spectral region between δ 160–50 ppm (Figure 40) was analyzed using DEPT–135 ^{13}C NMR experiments to confirm the chemical shifts for the CH_3, CH_2, and CH regions (Figure 41), and 2D NMR analysis was also performed (Figure 42). The assignment of the ^{13}C (Table 46) and the 2D NMR spectra were based on various literature references [48, 164, 174, 203-207].

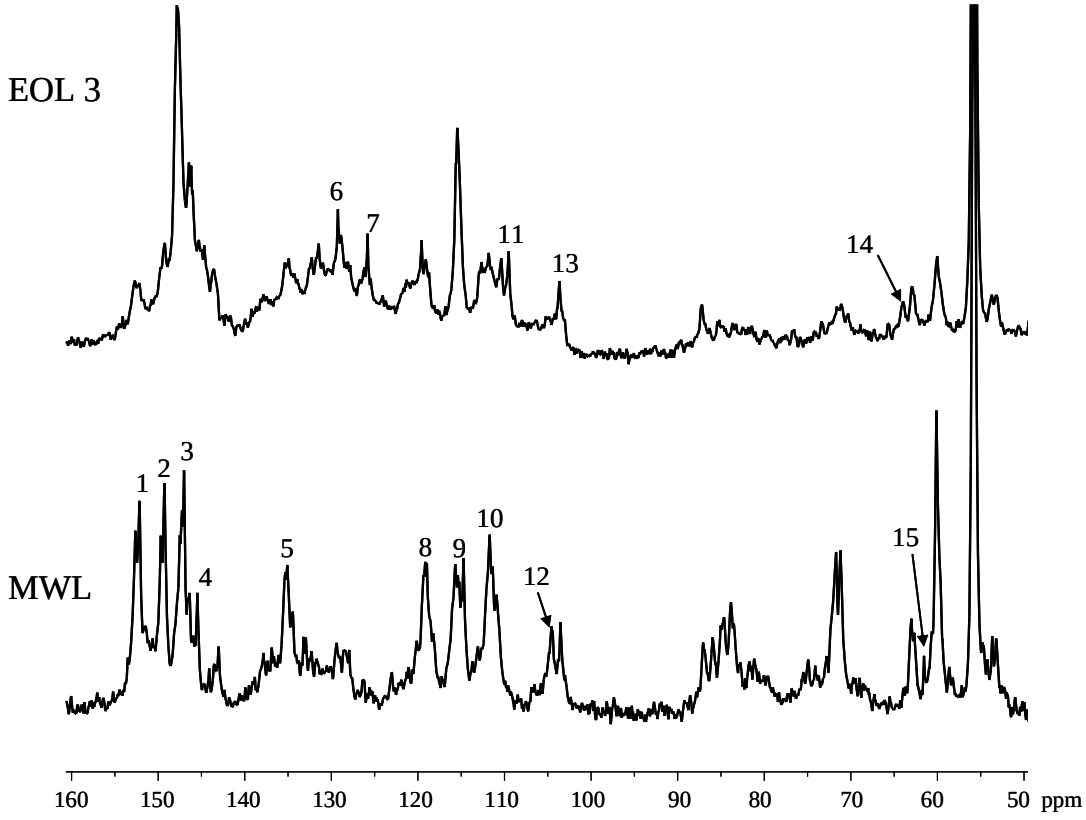


Figure 40. Expanded 160–50 ppm region of quantitative ^{13}C NMR spectra *B. davidii* MWL and EOL 3.

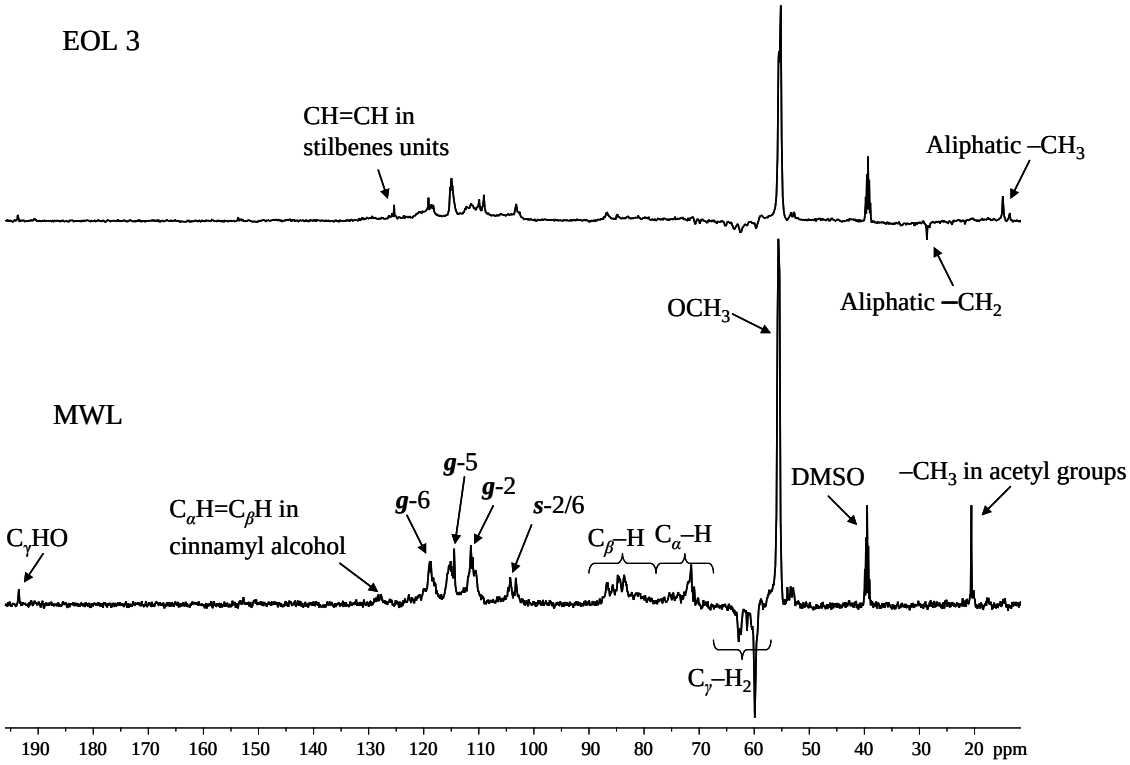


Figure 41. DEPT–135 ^{13}C NMR spectra of *B. davidii* MWL and EOL 3.

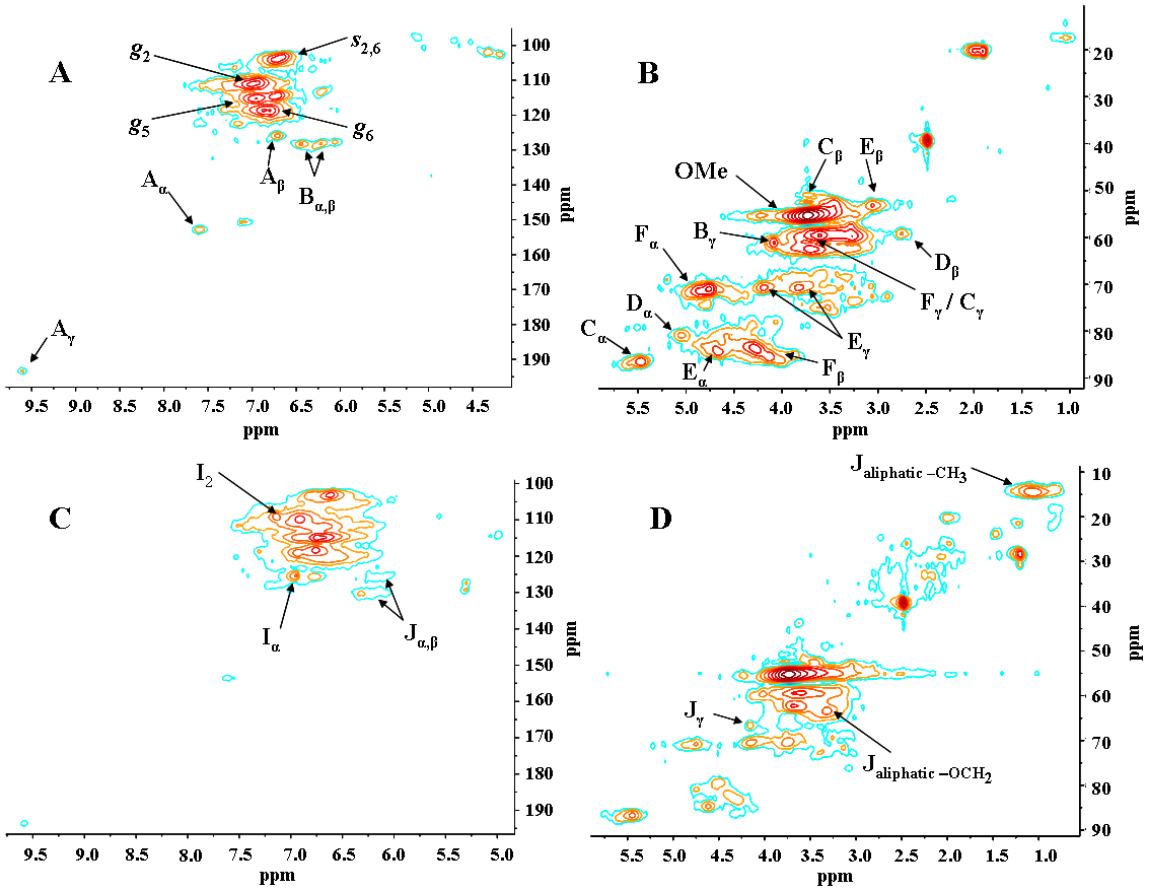


Figure 42. HSQC 2D NMR spectra of *B. davidii* MWL (A and B) and EOL 3 (C and D).

Table 46. Assignment of the expanded 160–50 ppm region of the quantitative ^{13}C NMR spectra.

Peak	Chemical Shifts (ppm)	Assignment
1	155 – 151	C_α in **A** & ***s***-3,5 in e[a] units, such as in **F**
2	151 – 148.5	***g***-3 in e units, such as in **F**
3	148.5 – 146.8	***g***-4 in e units & ***s***-3,5 in ne[b] units & ***g***-3 in ne units & ***g***-4 in conjugated C=C
4	145.5	***g***-4 in ne units, such as in **F**
5	136 – 134	***g***-1 in e units, such as in **F**
6	130 – 127	C_1 in **I** & C_1 and C_α in **J**
7	127 – 125	C_α in **I** & C_β in **I** and **J**
8	123 – 117	***g***-6 in e and ne units
9	117 – 114	***g***-5 in e and ne units
10	114 – 108	***g***-2 in e and ne units
11	109.5	***g***-2 in stilbene units, such as in **I**
12	108 – 104	***s***-2,6
13	103.6	***s***-2,6 in **E**
14	64	$–CH_2$ in Ethanol
15	61.6	C_γ in **B**

[a] e = etherified C_4; [b] ne = nonetherified C_4

The ^{13}C and DEPT NMR spectra showed that the intensity of the peaks in the spectral region between δ 90.0–58.0 ppm were diminished. Upon hydrolysis of *β-O*-4', significant loss in C_α–H, C_β–H, and C_γ–H was observed (Figure 40 and Figure 41), suggesting that these aliphatic carbons were cleaved and/or condensed. Li and Lundquist showed, based on model compounds, how *β-O*-4' units react under acid conditions [208]. One of the reaction pathways is that *β-O*-4' units undergo degradation to give stilbene structures (**I** in Figure 43). According to the NMR data (Figure 40, Figure 41, and Figure 42C), stilbene units (**I**) were detected in EOL giving rise to the $\mathbf{I}_\alpha$ signal at δ_C/δ_H 126/7.0 ppm. Such structures were also previously identified in Kraft lignin [209]. Equation 9 lays out the mechanism for the formation of stilbenes as depicted from published literature [208, 210].

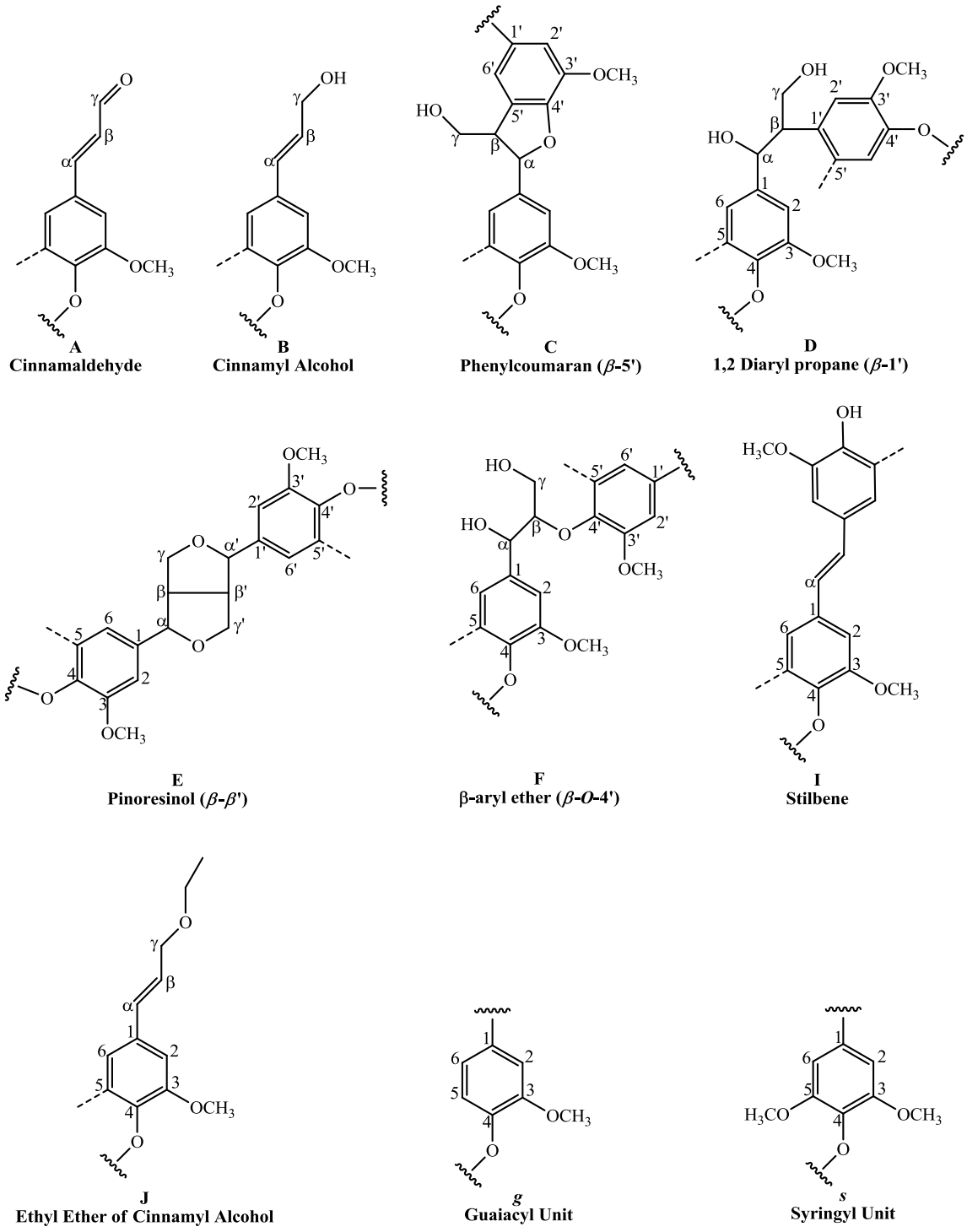
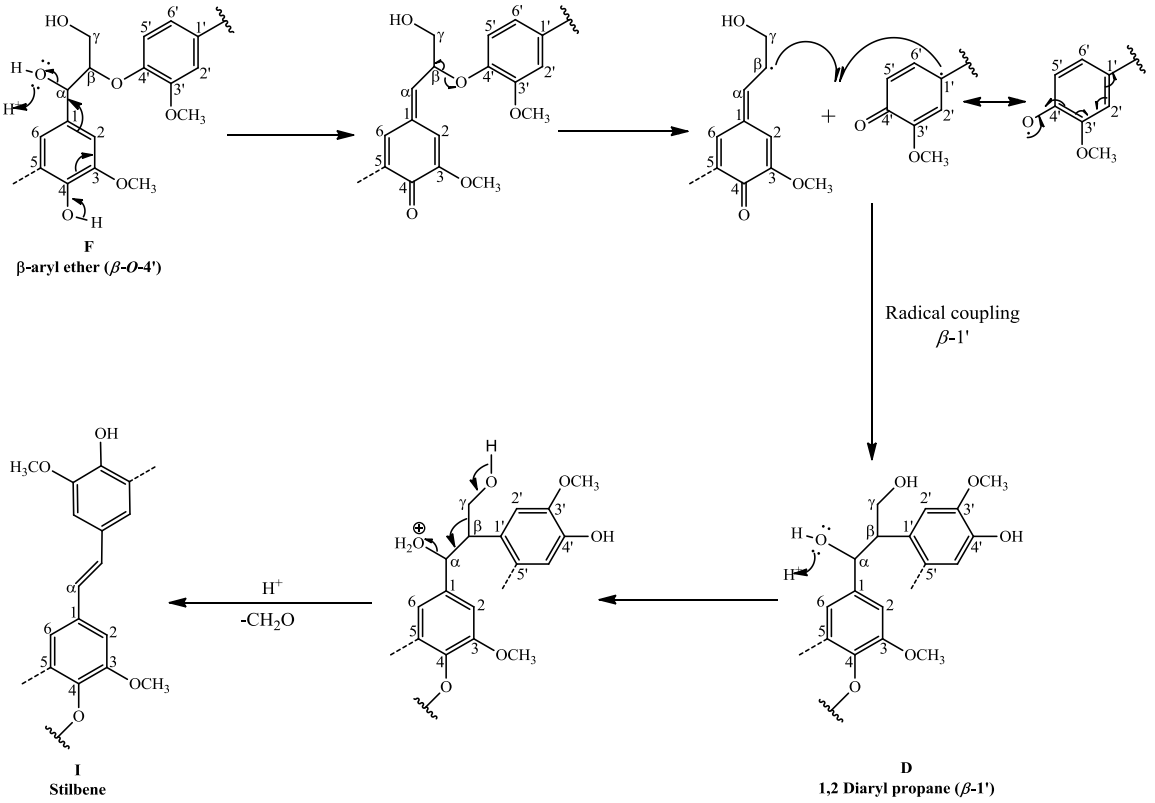


Figure 43. Lignin substructures identified in *B. davidii* MWL and EOL.



Equation 9. Degradation mechanism of *β*-aryl ether (*β-O*-4') during ethanol organosolv pretreatment.

The homolytic cleavage of *β-O*-4' via quinone methide intermediate causes the formation of *β*-1' interlinkage through radical coupling, which then in turn degrades under the acidic medium to give stilbenes through the loss of γ–methylol group as formaldehyde. The loss of the *β*-1' signals (**D**) (Figure 42B and D) after pretreatment is an indication that they were reactive and produced the stilbene structures. Therefore, both condensation and cleavage of aliphatic carbons occurred during EOP. The formation of stilbenes was also evident by the newly emerged peaks 6 and 11 in the ^{13}C spectrum of EOL 3 (Figure 40), where peak 6 and 11 are $\mathbf{I}_1$ and $\mathbf{I}_2$, respectively [207]. Radical coupling could also make *β*-5', *β-β'*, or 5-5' linkages (Figure 12) [208], but due to signal overlap such structures cannot be quantified individually. This radical coupling could

explain the increase in *β*-5' and/or *β*-*β′* content after pretreatment 1 (Table 45). On the other hand, Li and Lundquist reported that *β*-5' units can be converted to stilbenes through the same degradative pathway described in Equation 9, i.e. through the loss of γ–methylol group as formaldehyde [211]. Thus, the reduction of *β*-5' and *β*-*β′* content after pretreatment 2 and 3 (Table 45) could be attributed to *β*-5' degradation. Another reaction observed during ethanol organosolv pretreatment was the conversion of cinnamyl alcohol (**B**) to its ethyl ether (**J**), as Li and Lundquist also showed [208]. Cinnamyl alcohol disappeared after pretreatment, as indicated by the loss in peak 15, which corresponds to C_{γ} in **B**, and the signals of $C_{\alpha,\beta}$ in **B** in 2D at δ_C/δ_H 128/6.4–6.2 ppm (Figure 40, Figure 42A and C). The formation of the ethyl ether of cinnamyl alcohol is supported by peak 14 (–O–$\underline{C}H_2$–CH_3), which is the aliphatic –CH_2 in **J**, the aliphatic –CH_3 in **J** at 15.3 ppm, and the $\mathbf{J}_{\gamma}$ signal at δ_C/δ_H 67/4.2 ppm (Figure 40 and Figure 42D).

The reduction in the intensities of peaks 1, 2, and partially for 3 is indicative of aryl-*O*-ether (*β*-*O*-4') linkage scission (Figure 40), since the content of ***s***-3,5 and ***g***-3,4 in etherified units decreases, while the amount of ***s***-3,5 and ***g***-3 in nonetherified units increases (Table 46). The quantitative data in Table 45 suggested that the content of the oxygenated aromatic region did not change because the decrease in peaks 1 and 2 was compensated by the increase in spectral region at δ 149.0–146.8 ppm. This increase was not only due to the homolysis of *β*-*O*-4' but also to the formation of stilbene structures. The signals of $\mathbf{I}_{3,4}$ fall in the region of peak 3 (δ 148.5–146.8 ppm), where $\mathbf{I}_3$ and $\mathbf{I}_4$ have chemical shifts at δ 147.8 ppm and δ 146.3 ppm, respectively [207]. Typically, ***g***-4 has a chemical shift at δ ~145.5 ppm (peak 4); however, the addition of a conjugated C=C on a guaiacyl unit shifts the signal of this carbon downfield to δ 146.3 ppm, which resulted in

a decrease in peak 4 (Figure 40). In the aromatic C–C region, peak 5 ($\mathbf{F}_1$) decreased as a result of *β-O-*4' cleavage. More accurately, the signal of this carbon was shifted upfield to correspond to $\mathbf{I}_1$, resulting in an increase at δ 129.2 ppm [207].

The shape of peak 9 after pretreatment appeared to increase, although the quantitative data suggested otherwise. This peak corresponds primarily to ***g***-5 in etherified and nonetherified units [204]. After pretreatment, the peak became sharper and distinct indicating a change in its structural environment. Since most of the ether linkages were broken during pretreatment, this peak corresponds mainly to ***g***-5 in nonetherified units. In the case of syringyl lignin, there are two peaks assigned to the ***s*** units, peak 12 and 13. Peak 12, which is ***s***-2,6 in general, decreased after pretreatment, but ***s***-2,6 in *β-β'* (peak 13) did not obviously change, implying that such substructure was recalcitrant to degradation during ethanol organosolv pretreatment.

6.3.4 Structural Analysis of *B. davidii* EOL Using Quantitative ^{31}P NMR

The most abundant linkage in lignin is the aryl-*O*-aliphatic interlinkage, *β-O-*4' [2]. As observed from the ^{13}C NMR data, the cleavage of this *β*-ether bond was facile and increased with increasing pretreatment severity yielding the corresponding phenolics. The amount of phenolic hydroxyl groups before and after pretreatment was determined using lignin phosphitylation and quantitative ^{31}P NMR. This technique is based on the selective phosphitylation of the hydroxyl groups using 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP) (Equation 7) [162]. Figure 44 presents the quantitative ^{31}P NMR spectrum of *Buddleja davidii* MWL [187] and EOL 3. The concentrations of the hydroxyl groups were calculated based on the internal standard (i.e. cyclohexanol) and the respective integrated peak area (Table 47).

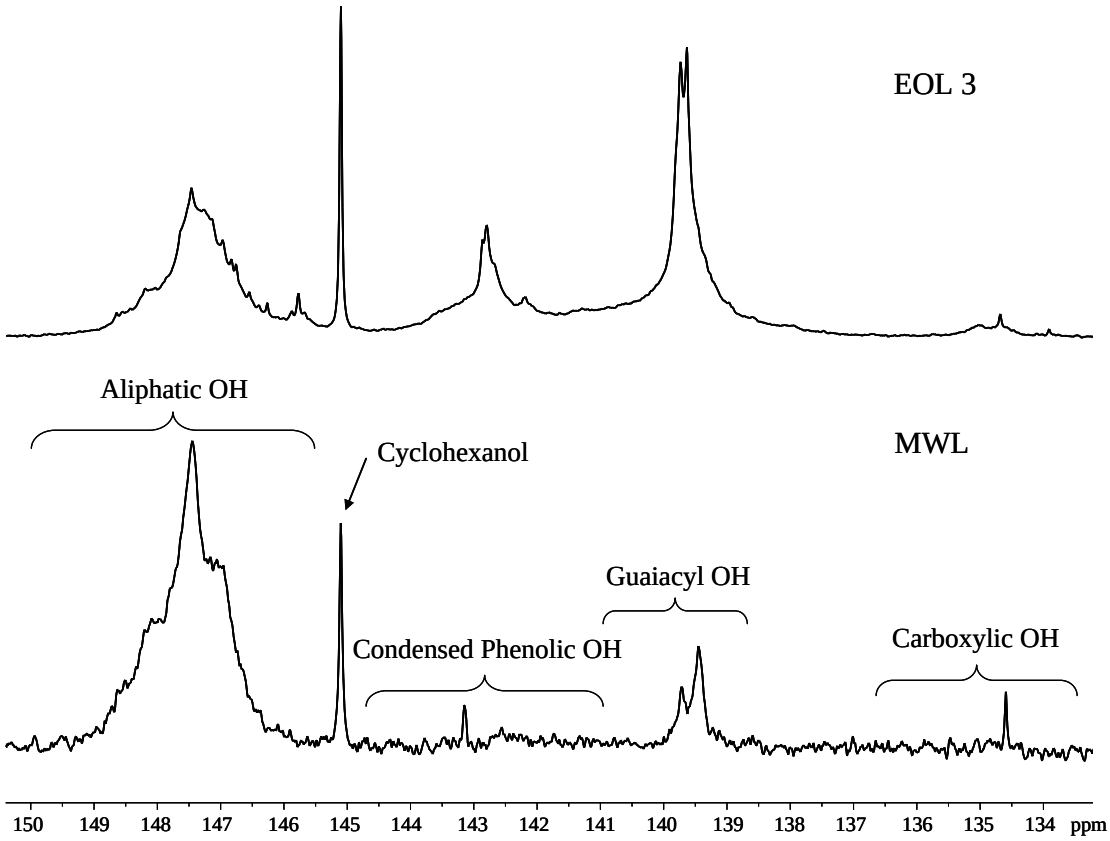


Figure 44. Quantitative ^{31}P NMR spectra of *B. davidii* MWL and EOL 3.

Table 47. Hydroxyl content of MWL and EOL of *Buddleja davidii* as determined by quantitative ^{31}P NMR

	Hydroxyl Content (mmol/g lignin)			
	Aliphatic OH (δ 150.0 – 145.5)	Condensed Phenolic OH (δ 144.7 –141.0)	Guaiacyl OH (δ 141.0 – 138.8)	Carboxylic OH (δ 136.6 – 133.6)
MWL [187]	4.51	0.27	0.43	0.03
EOL 1	2.67	0.98	1.66	0.16
EOL 2	2.51	0.98	1.53	0.17
EOL 3	1.86	1.07	1.66	0.15

The ^{31}P NMR data was consistent with the proposed homolysis of *β-O*-4' units and related aryl-*O*-aliphatic linkages. The amount of phenolic OH, both condensed and guaiacyl, increased significantly after pretreatment. The concentration of condensed

phenolic OH increased from 0.27 mmol/g lignin in MWL [187] to 1.07 mmol/g lignin in EOL 3. Guaiacyl phenolic OH increased from 0.43 mmol/g lignin in MWL [187] to 1.66 mmol/g lignin in EOL 3. This data supports the decrease in peak 1 (***s***-3,5 etherified) and peak 2 (***g***-3 etherified) as well as the increase in peak 3 (***s***-3,5 ***g***-3 nonetherified, $\mathbf{I}_3$, and $\mathbf{I}_4$) in the ^{13}C NMR data (Figure 40). The increase in phenolic OH could also be due to the demethylation of lignin (Table 45) to form catechol. On the contrary, the content of aliphatic OH (dominant group) after pretreatment decreased by 41% – 59% with respect to the MWL. This was attributed to the loss of γ–methylol group as formaldehyde and OH groups on $C_{\alpha,\beta}$, or the whole side chain in general to form *β*-1' linkages, which then were converted to stilbene structures. Also, oxidation of aliphatic OH might have occurred as supported by the significant increase in the carboxylic OH content (Table 47). The increase in phenolic hydroxyl groups and the decrease in aliphatic hydroxyl groups are desired features for potentially using *B. davidii* EOL as an antioxidant [135].

6.4. Conclusions

^{13}C and ^{31}P NMR techniques were used to study the structure of *Buddleja davidii* EOL in order to understand the delignification mechanism of ethanol organosolv pretreatment (EOP) and the recalcitrance of lignin. EOP was capable of efficiently delignify *B. davidii* biomass. GPC results showed significant decrease in the molecular weight of lignin (from DP ~80 to DP ~12). Such decrease was primarily due to the extensive homolytic cleavage of *β*-*O*-4' interlinkages as indicated by the quantitative ^{13}C NMR data. The amount of *β*-*O*-4' linkages decreased by 57% after pretreatment 3. The breakage of this bond led to the formation of stilbene structures, loss of aliphatic side-

chain, as well as increase in the degree of condensation (82% in EOL 3). Stilbene units were clearly identified in the 2D HSQC NMR spectrum. On the other hand, *β*-5' and *β*-*β*' substructures were resistance to degradation during EOP. Acid-catalyzed conversion of cinnamyl alcohol to its ethyl ether was another reaction that took place during pretreatment. The reduction in guaiacyl and syringyl lignin suggested that they are both reactive toward condensation.

In summary, *B. davidii* EOL possesses several qualities that make it suitable for different co-product applications, including low oxygen content, molecular weight, and aliphatic OH as well as high phenolic OH. EOP seemed to be selective in terms of bond cleavage and its ability to delignify lignocellulosic materials. The pretreatment was capable of degrading certain linkages, such as *β*-*O*-4', *β*-1', and slightly *β*-5' to form stilbene structures. Through cleavage of these bonds, it produced lignin chains with DP sufficiently low to become soluble in ethanol. Condensation was not found to be a counterproductive process, since the increase in DC values did not hinder the delignification efficiency of EOP. Although lignin is recalcitrant in its nature, it mainly contains *β*-*O*-4' linkages that can be easily degraded in EOP. If enough of this bond was cleaved, the lignin polymer would be broken into short chains readily soluble in ethanol.

CHAPTER 7

CORRELATION BETWEEN ANATOMICAL CHARACTERISTICS OF ETHANOL ORGANOSOLV PRETREATED *BUDDLEJA DAVIDII* AND ITS ENZYMATIC CONVERSION TO GLUCOSE[4]

7.1 Introduction

The development of alternative low-carbon transportation fuels is an urgent global priority due to increasing demand for energy, diminishing petroleum reserves, and climate change [1, 15, 16]. Biofuels produced from lignocellulosic biomass have been recognized as a potential sustainable source of such energy [1, 16]. In the early stages of the cellulosic ethanol industry, one step concentrated acid, such as H_2SO_4 or HCl hydrolysis was used to convert biomass to ethanol [138]. An alternative to the acid route is the bioconversion process of lignocellulosic materials into bioethanol, which involves pretreatment, enzymatic hydrolysis, and fermentation [7]. Pretreatment is considered to be the crucial step because it governs the susceptibility of the biomass to the downstream hydrolysis and fermentation [8, 10]. However, the exact effects of pretreatment on the biomass are not thoroughly understood and they are dependent on both the type of biomass and its interaction with the pretreatment methodology used [143]. The

[4] This manuscript was accepted for publication in Biotechnology and Bioengineering, 2010. It is entitled as "Correlation between Anatomical Characteristics of Ethanol Organosolv Pretreated *Buddleja davidii* and its Enzymatic Conversion to Glucose". The other authors are Arthur J. Ragauskas from the Institute of Paper Science and Technology and School of Chemistry and Biochemistry at Georgia Institute of Technology and Michael Ray and Richard J. Murphy, from the Division of Biology at Imperial College London, UK.

processing of lignocellulosic biofuels via the enzymatic hydrolysis route starts with the pretreatment of the biomass [7]. The purpose of the pretreatment is to overcome the effects of biomass recalcitrance so that cellulose can become more amenable to enzymatic hydrolysis [1]. The consensus in the literature is that crystallinity of cellulose, its degree of polymerization (DP), lignin content/structure, lignin-carbohydrate complexes, hemicellulose content, surface area, and pore size are the main factors that affect the enzymatic digestibility [70, 145, 148]. A pretreatment technology of interest in the present work is ethanol organosolv pretreatment, in which the biomass is treated in an ethanol, sulfuric acid, and water mixture [129, 132, 134]. This pretreatment technology has been shown to be effective on both hardwood and softwood, as high enzymatic conversion of cellulose-to-glucose (90 – 100%) was achieved [129, 132]. Another desirable feature of organosolv pretreatment is its ability to isolate lignin as a solid material with characteristics that can be used in various co-products [130]. In previous work, we demonstrated that enhanced enzymatic saccharification in ethanol organosolv pretreated *Buddleja davidii* biomass was accomplished by the removal of hemicellulose and lignin, reduction in the DP of cellulose, and conversion of the stable crystalline cellulose dimorphs (I_α/I_β) to the easily degradable *para*-crystalline and amorphous celluloses [194]. *B. davidii* is a shrub that originated in china but has been naturalized in different parts of the world, including parts of the United Stated and Europe [187]. It exhibits a very wide range of growth habitat; it is perennial and grows up to 3-5 m high [187]. The biomass composition of *B. davidii* is 30% lignin, 35% cellulose, and 34% hemicelluloses [187]. This biomass is currently being examined as a source for value-added chemicals, such as anti-inflammatory and antioxidant compounds [31]. If this

bioresource would develop into a crop for the purpose of extracting such chemicals, then the rest of the biomass could be used as a potential feedstock for biofuels production. This study provides information on the anatomical characteristics of the pretreated *B. davidii* biomass after ethanol organosolv pretreatment. Improving our fundamental knowledge of pretreatment technologies will lead to significant advances in the field of sustainable low-cost cellulosic biofuel production.

7.2 Experimental Section

7.2.1 Preparation of Biomass

Buddleja davidii stems were obtained from a single plant from a garden in Georgia, USA in September 2008 and prepared as described in Chapter 3 (3.1.2.2 *Buddleja davidii* for Ethanol Organosolv Pretreatment Experiment). The wood was ground and ethanol organosolv pretreated as described in Chapter 3 (3.2.4 Ethanol Organosolv Pretreatment). Three pretreatment conditions were performed as follows: condition 1 was carried in 1.25 % w/w H_2SO_4 and 50 % v/v ethanol at 180 °C for 60 min; condition 2 was carried in 1.75 % w/w H_2SO_4 and 50 % v/v ethanol at 180 °C for 40 min; condition 3 was carried in 1.50 % w/w H_2SO_4 and 65 % v/v ethanol at 195 °C for 60 min.

7.2.2 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed as described in Chapter 3 (3.3.3 Enzymatic Hydrolysis).

7.2.3 Embedding

The pretreated ground wood (approx 5 mm particle size) was embedded in an acrylic resin (LR White) prior to sectioning, as previously described [212]. The following embedding procedure was used: samples were fixed for 12 h in formalin-acetic acid-alcohol (FAA), which contains 63% ethanol, 4% glacial acetic acid, and 6% formaldehyde (from a 37-48% solution). The ground wood was dehydrated in a graded ethanol series of 70, 80, 95, and 2 x 100% for thirty minutes at each application. Increasing the concentration of LR White in ethanol up to 100% gave adequate infiltration. Each substitution was left for 2 hours in a rotator. The concentrations of LR White in ethanol were 20, 40, 60, 80, 90, and 100%. Samples were added to size 4 Agar gelatin capsules and completely filled with fresh LR White resin, capped and placed in an oven set at 60°C for 24 h to polymerize the resin.

7.2.4 Sectioning and Staining

The gelatin capsules were removed and the polymerized specimens were sectioned using freshly cut glass blades in a rotary microtome set at 2 μm thickness. Sections were then stained with 1% (w/v) aqueous safranin for 2 min, briefly washed with deionized water and permanently mounted in di-n-butyl phthalate in xylene (DPX).

7.2.5 Light Microscopy Imaging and Analysis

Prepared slides were examined under bright field illumination using a Leitz Diaplan microscope, and the micrographs were taken using a Nikon CoolPix 990 digital camera. The analysis was based on a semi-quantitative technique. Ten wood particles from the untreated (controls) and pretreated samples were carefully examined under the

microscope using the x40 and x100 objective lens, and the cells were classified in a visual rating system that consists of three features: middle lamella integrity, cell shape, and cell wall continuity (Figure 45). The error bars in Figure 46a represent the standard deviations calculated from the sample size n=10.

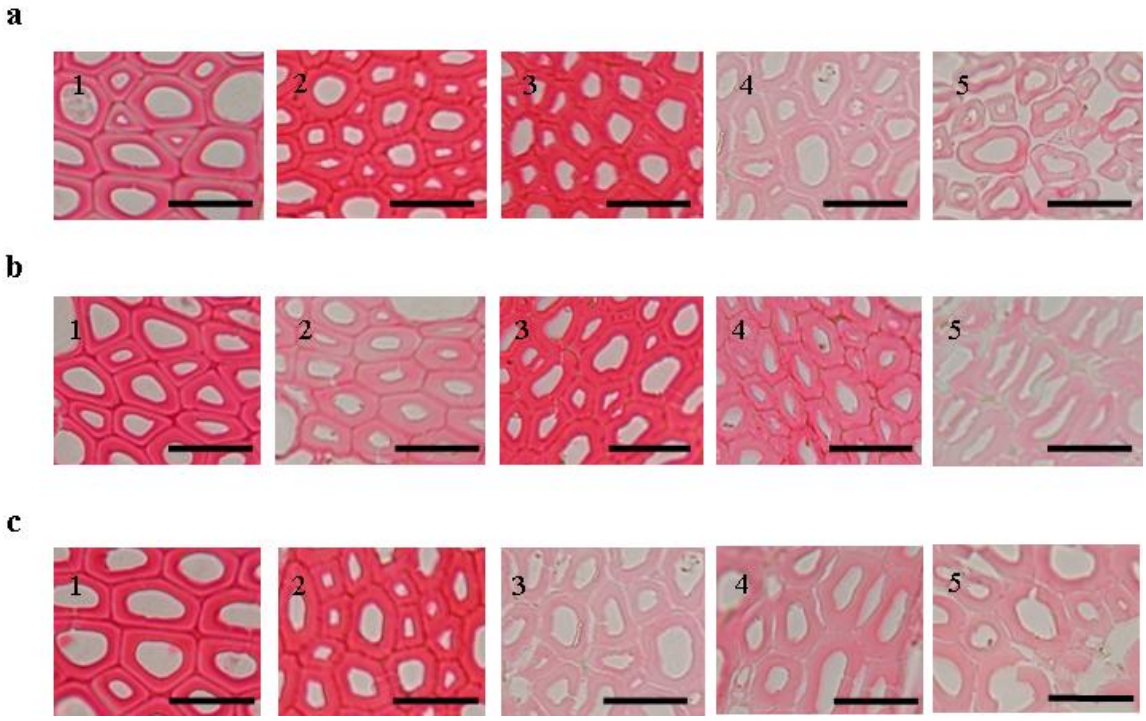


Figure 45. Rating system used to categorize the cell structural features of untreated and pretreated *B. davidii*. a, Middle Lamella (ML) integrity feature, where 1 = ML is unaffected, clear, and discrete; 2 = ML shows signs of distortion; 3 = ML shows irregularities, deformations, and a crinkly appearance; 4 = ML is partially removed; 5 = ML is mainly removed. b, Cell shape feature, where 1 = cell is regular with sharply defined lumen; 2 = cells are shrinking; 3 = cells are slightly deformed; 4 = lumen is rough and disrupted; 5 = cells are heavily distorted. c, cell wall continuity feature, where 1 = cell wall is sharply defined and continuous with clear pits; 2 = cell wall is rough and swollen; 3 = cell wall is uneven and distorted; 4 = few cracks appear in the cell wall; 5 = multiple cracks and broken cell wall. Scale bar, 20 μm.

7.3 Results and Discussion

In this study, we examined the cellular structure of *B. davidii* after ethanol organosolv pretreatment to further understand the effects of this pretreatment approach on the cell wall structure of the biomass. The pretreated biomass showed changes in three of its cellular features when compared with the untreated controls: middle lamella (ML) integrity, cell shape, and cell wall continuity. We developed a visual rating system to assess these features in micrographs of ethanol organosolv pretreated *B. davidii* as described in Figure 45. The average ratings are presented in Figure 46a.

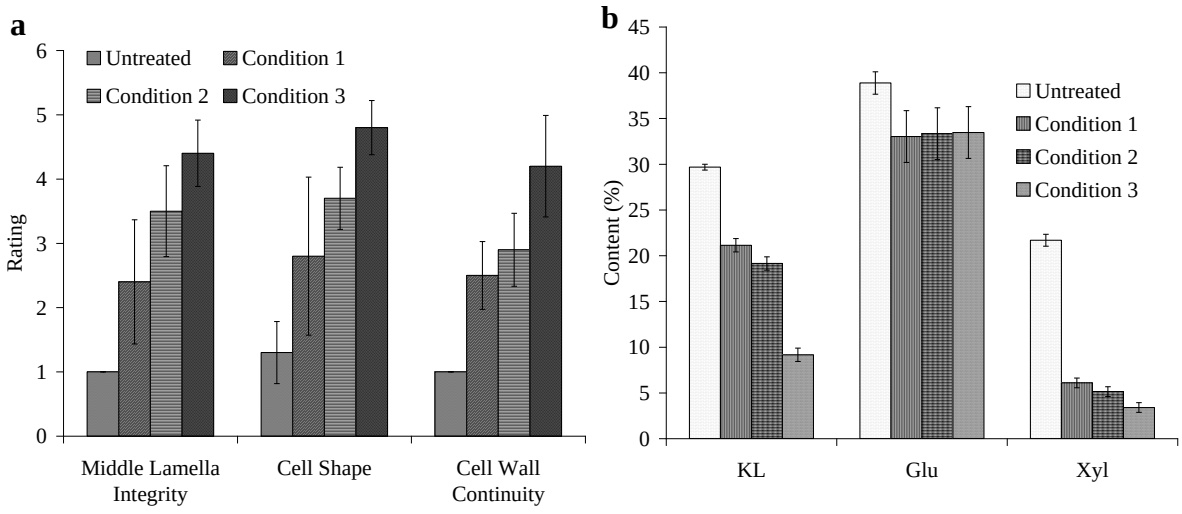


Figure 46. Quantitative representation of the characteristics of the untreated and pretreated *B. davidii*. a, Average ratings based on the criteria outlined in Figure 45. b, Klason lignin (KL), glucose (Glu), and xylose (Xyl) contents measured as % (w/w) in oven-dried untreated wood. Error bars are the standard deviations [194].

The anatomical characteristics of three pretreated *B. davidii* biomass samples produced under various pretreatment conditions that gave differing compositional and/or enzymatic hydrolysis profiles (Figure 46b and Figure 47) [194], were investigated. Figure 46b shows the amount of Klason lignin, glucose, and xylose in the untreated and ethanol

organosolv pretreated biomass. The three pretreatment conditions removed lignin and hydrolysed hemicelluloses to differing extents. However, the glucose yield (or recovery, ~85%) remained broadly the same among the different pretreatment conditions. Following enzymatic saccharification, condition 1 gave relatively low cellulose-to-glucose conversion (68%), whilst conditions 2 and 3 gave high conversion levels, 96% and 98% respectively (Figure 47) [194].

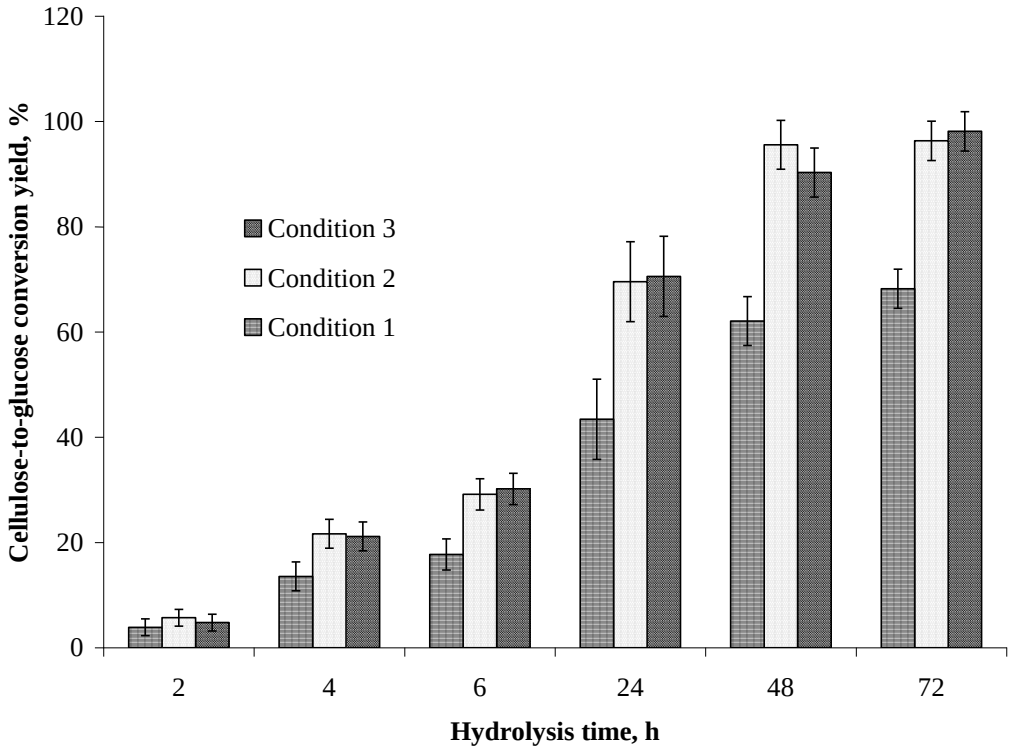


Figure 47. Enzymatic conversion of *Buddleja davidii* cellulose to glucose after ethanol organosolv pretreatment carried under different conditions [194].

The same untreated and previously ethanol organosolv pretreated biomass materials [194] were then microscopically studied. Untreated biomass presented a baseline level in the rating system for the three features, representing 'native' ML, cell shape, and cell wall continuity (Figure 48a). As the pretreatment severity increased

moving from pretreatment condition 1 to 3, these ratings increased as well. Having moderate ratings, condition 1 showed no obvious removal of ML but some irregular appearance and distortions in the ML were observed (Figure 48b). Condition 1 also caused the cell wall to swell and introduced distortion at the cell wall/cell lumen boundary (Figure 48b and c). Selective delignification of the ML was observed after pretreatment conditions 2 and 3, as indicated by the absence of ML (pointed out in Figure 48d and e). The ML was partially removed after pretreatment condition 2, while pretreatment condition 3 caused complete ML removal and consequently separation of the individual cells. Selective delignification has also been observed after biomechanical pulping and biodegradation of biomass, where gradual lignin loss from the lumen towards the ML was reported [213, 214]. Under condition 2, the inner part of the cell wall (nearest the lumen) became rough and the cell wall became uneven and distorted (Figure 48c). The most severe pretreatment, condition 3, caused significant damage to the cells including extensive distortion, cracking, and breaks in the cell wall (Figure 48e and f).

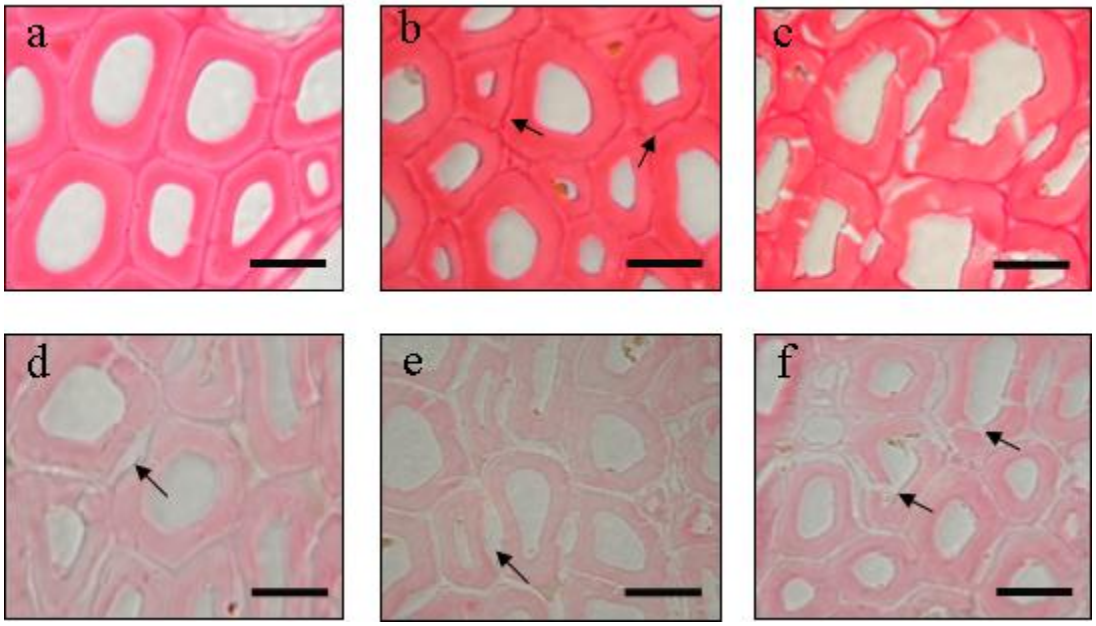


Figure 48. Effect of ethanol organosolv pretreatment on the cell structure of *Buddleja davidii*. a, Normal cells with smooth unaffected middle lamella, regular shape, continuous wall, and clear pitting. b, The arrows point to the development of an irregular appearance in and some distortion of the ML. The cell wall is swollen. c, Cells are deformed, the lumen is rough and disrupted, and the cell wall is distorted and uneven. d, Partial removal of the ML indicating delignification which is also evident by the lighter safranin staining. e, Middle lamella is mainly removed and cells are heavily distorted. f, Cracks and broken areas appear in the cell wall as indicated by the arrows. Scale bar, 10 µm.

Figure 46b shows that condition 3 removed more lignin and hemicelluloses than condition 1 and 2. A direct relationship has been demonstrated in several studies between lignin removal from the biomass and the extent of subsequent enzymatic hydrolysis [123, 127, 145, 186, 190]. However, in the present and other studies with organosolv pretreatment, the efficiency of enzymatic digestion was not always directly proportional to lignin content [130, 132, 194]. The biomass produced after pretreatment condition 1 and 2 had similar lignin contents (Figure 46b) but very different degrees of cellulose-to-glucose conversion (Figure 47) [194]. Furthermore, pretreatment condition 2 and 3 produced solids that exhibited similar degrees of enzymatic release of glucose but at very

different lignin contents. The anatomical study of the untreated and variously pretreated *B. davidii* provides useful insights on the lignin removal effects that could be related to the differences observed in saccharification behavior.

Partial removal of lignin from the middle lamella regions of organosolv pretreated *B. davidii* was sufficient to substantially enhance the enzymatic conversion of cellulose-to-glucose. This is supported by the fact that the enzymatic hydrolysis of *B. davidii* produced under pretreatment condition 2 was very effective (96% cellulose-to-glucose conversion) even though the biomass contained similar amount of lignin compared to that produced under pretreatment condition 1 (~19% Klason lignin). We hypothesize that partial delignification permits cellulases to attack cellulose not only from the lumen side of the cell wall but also from the ML region due to its disruption or removal. In contrast, pretreatment condition 1 did not cause extensive disruption of the ML and thus the resulting biomass was less readily digested by cellulases (68% cellulose-to-glucose conversion). Therefore, these results emphasize that the efficiency of the enzymatic hydrolysis is not dependent only on the lignin content but also on its physical distribution in the biomass matrix. A similar conclusion was drawn in the study done by Donohoe *et al*. where they mentioned that the re-localization of lignin and its movement away from the cellulose microfibril surfaces, without being removed from the biomass, improve cellulase accessibility [215]. Pretreatment condition 3 generated *B. davidii* biomass that was fully hydrolysed enzymatically (97% cellulose-to-glucose conversion); however, it was a more severe pretreatment than condition 2, suggesting that further delignification and more or less complete removal of ML lignin beyond that achieved via pretreatment 2 is unnecessary for complete enzymatic saccharification. Indeed, a study by Ishizawa *et al*.

showed that significant reduction in the lignin content in the biomass (< 5%) can negatively affect cellulose digestibility because cellulose microfibrils tend to aggregate in the absence of lignin, resulting in decreased cellulase accessibility [216]. Also, these observations imply that heavy distortion and/or breaking of the cell wall are not necessary for efficient enzymatic hydrolysis. Using less severe pretreatment conditions is advantageous because it minimizes processing energy, chemical inputs, and the generation of fermentation inhibitors (furfural and 5-hydroxymethyl furfural (HMF)) produced from sugar degradation [136].

We have shown previously that the crystallinity index of *B. davidii* cellulose does not change significantly during pretreatment, where it slightly decreased from 0.55 to 0.49 after pretreatment condition 3 [194]. This observation is clearly supported by the polarized images shown in Figure 49, where the S1 layer of the secondary cell wall is exhibiting strong birefringence indicating crystalline cellulose. The high level of delignification accomplished by pretreatment condition 3 (~64%) which drastically affected the lignin-rich ML regions, as indicated by the separation of cells and dissolution of ML, did not drastically disrupt the cellulose crystallinity (Figure 49). However, the ultrastructure of cellulose changed during pretreatment condition 3, as previously demonstrated, to give less cellulose I_α and I_β but more *para*-crystalline and amorphous cellulose [194].

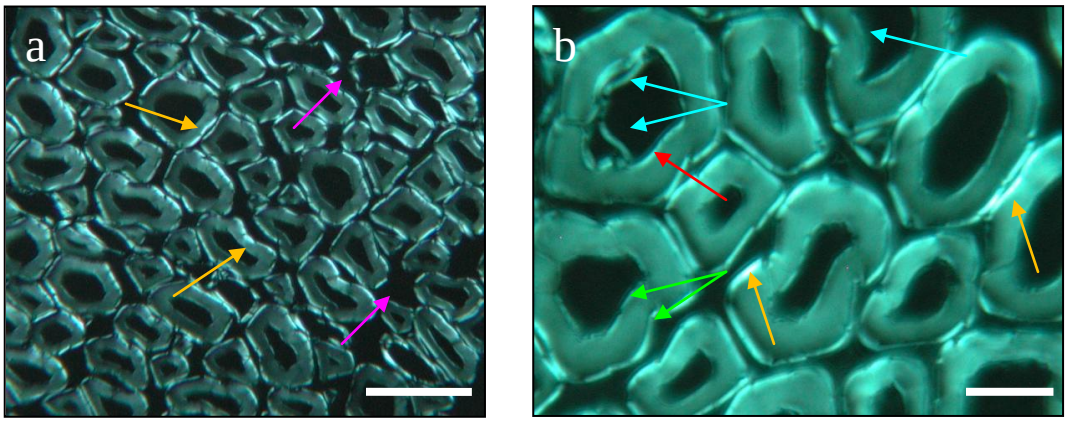


Figure 49. Polarized light images of ethanol organosolv pretreated *Buddleja davidii* under condition 3. In both images, cellulose orientation in S1 layer of cell wall exhibits strong birefringence indicating crystalline cellulose orientated perpendicular to the transmitted light (yellow arrows). a, Shows separation of cells and dissolution of middle lamella region (pink arrows). Scale bar, 20 µm. b, Shows hints of a birefringent S3 layer (very thin) (red arrow). There are suggestions of small dislocations in the S1 and S3 layers (green arrows). Cell walls also show disruptions, kinks and indications of delamination (blue arrows). Scale bar, 10 µm.

The degradation of the middle lamella caused by the organosolv pretreatment was the most striking and distinctive feature of the microscopic observations. Lignin is known to be the most recalcitrant biopolymer in the biomass and is found at high concentration in the ML [217]. Typically, lignin in the ML of hardwoods has a higher content of guaiacyl (***g***) units than syringyl (***s***) units (low ***s***/***g*** ratio) [217]. It can be predicted that it is more difficult to hydrolyse this lignin since there is the opportunity of having a higher content of covalent C-C crosslinking between guaiacyl units. Yet, ethanol organosolv pretreatment was capable of partially or completely removing the lignin in ML in *B. davidii* wood. The milled wood lignin of this plant species has both guaiacyl and syringyl lignins with an ***s***/***g*** ratio of 19:81 [187].

7.4. Conclusions

The anatomical features of *Buddleja davidii* subject to different severities of ethanol organosolv pretreatment were investigated in order to gain further insights to explain observed differences in the amounts of glucose released in enzymatic saccharification reactions. The features that varied with pretreatment severity were: middle lamella integrity, cell shape, and cell wall continuity. The removal of hemicellulose and lignin was important for improving enzymatic hydrolysis, as indicated by the cellulose-to-glucose conversions yielded after the three pretreatment conditions. However, the absolute amount of lignin remaining in the biomass after pretreatment was not a major factor dictating the extent of enzymatic glucose release under the conditions studied, as concluded from the enzymatic saccharification after pretreatment condition 1 and 2. Indeed, it appears that the physical distribution of lignin within the biomass matrix after pretreatment is a key factor influencing enzymatic hydrolysis. Certainly, the removal of lignin from the middle lamella regions of the biomass tissue structure due to the organosolv pretreatment is another important factor increasing the efficiency of cellulose hydrolysis presumably due to enhanced enzyme access. Thus, the chemical and physical changes that occur to the biomass after pretreatment are what dictate efficient enzymatic digestibility. Such changes include: decreasing the DP of cellulose, lignin content, hemicellulose content, as well as altering the biomass cell wall structure.

CHAPTER 8

OVERALL CONCLUSIONS

The first thesis study (chapter 4) started with the idea of investigating *Buddleja davidii* as a potential feedstock for bioethanol production. *B. davidii* was of interest because it has several attractive agro-energy features. The plant was analyzed for its ash, extractives, cellulose, hemicelluloses, and lignin contents, as well as detailed structural elucidation of lignin and cellulose. The ash content in the leaves, bark, and stem were 6.30%, 4.75%, and 0.73%, respectively. The main inorganic elements in the leaves, bark and stem were K, Ca, P, S, and Mg. The importance of such information emerges from the fact that these nonprocessed elements represent a waste stream that will need to be addressed in an environmentally acceptable manner, which in turn adds to the production/capital cost. The extractives were 3.22%, 2.83%, and 0.30% in the leaves, bark, and stem, respectively. Extractives are high-value added chemicals that should be extracted prior to pretreatment. The types of extractives found were: aromatics, alkanes, fatty acids, alcohols, and sterols. The composition of *B. davidii* stem was 30% lignin, 35% cellulose, and 34% hemicelluloses. Compared to other softwoods and hardwoods, *B. davidii* appeared to have high lignin and hemicellulose and low cellulose. Pretreatment employed for this plant must be capable of hydrolyzing such high content of lignin and hemicelluloses, while preserving cellulose, in order to produce pretreated biomass that can be readily ezymatically hydrolyzed. Cellulose crystallinity was relatively high (0.55), but the DP was relatively low (1000). High cellulose crystallinity could impede cellulose crystallinity but low DP would favor efficient enzymatic hydrolysis. *B. davidii* lignin

contains both guaiacyl and syringyl units with a ratio of 81:9 ***g:s***. The main interlinkage is *β-O*-4' and the degree of condensation is 45%. The dominant OH group in the lignin is at aliphatic sites. The lignin has considerable amount of free guaiacyl OH, but low carboxylic acid OH. The lignin information was later used in Chapter 6 to fundamentally understand the chemical transformations of lignin during EOP.

To further evaluate *B. davidii* as a potential feedstock for bioethanol production, ethanol organosolv pretreatment was performed as described in Chapter 5. The biomass was pretreated and delignified, while 85% of the glucose content of the untreated material was retained in the pretreated solid fraction. The enzymatic hydrolysis showed that EOP produced solid substrates that were readily digestible by cellulases (~98% conversion of cellulose to glucose). Thus, the presence of high lignin and hemicelluloses contents in *B. davidii* wood was not found to be a negative factor since ~50% of these polymers were hydrolyzed/solubilized. In the second part of the study in Chapter 5, the fundamental characteristics of cellulose after pretreatment were analyzed by GPC and CP/MAS ^{13}C NMR spectroscopy. Cellulose DP was determined by GPC and NMR was used to study the changes in crystallinity and ultrastructure of cellulose. The results showed a decrease in DP along with an increase in the relative proportions of *para*-crystalline and amorphous cellulose and a decrease in cellulose I_α and I_β. The main conclusion from this study was that the removal of lignin and hemicellulose, reduction in DP, and decrease in the crystalline allomorphs (I_α and I_β) increased the amenability of the biomass to enzymatic degradation.

In Chapter 6, detailed chemical structural elucidation of EOL of *Buddleja davidii* was performed to determine the fundamental structure released from an EOP. Several

nuclear magnetic resonance (NMR) techniques were used to analyze the structure of EOL, including quantitative ^{13}C, ^{31}P NMR, and qualitative DEPT-135 ^{13}C, 2D ^{1}H-^{13}C correlation NMR. As revealed by NMR, the aryl-O-ether linkage (*β-O*-4') of lignin was extensively cleaved via homolysis during pretreatment and led to the formation of stilbene structures. Other linkages, such as resinol (*β-β'*) and phenylcoumaran (*β*-5'), were resistant to degradation. The high degree of condensation of EOL indicated that condensation reactions occurred but did not impede the delignification efficiency of EOP. Both guaiacyl and syringyl lignin were found to be reactive toward condensation during pretreatment. The results from GPC showed that the degree of polymerization (DP) of lignin significantly decreased by ~85%, facilitating lignin solubilization in ethanol.

In the last chapter in the thesis, the anatomical characteristics of *B. davidii* were investigated before and after EOP in order to further understand the alterations that occur to the cellular structure of the biomass which can then be correlated with its enzymatic digestibility. Results showed that the EOP of *B. davidii* selectively removes lignin from the middle lamella (ML), which does not significantly disrupt the crystalline structure of cellulose. The removal of ML lignin is a major factor in enhancing enzymatic cellulose-to-glucose hydrolysis. The pretreatment also causes cell deformation, resulting in cracks and breaks in the cell wall. These observations, together with characterization analysis of the cell wall polymer material, lend support to the hypothesis that the physical distribution of lignin in the biomass matrix is an important structural feature affecting biomass enzymatic digestibility.

Overall, *Buddleja davidii* is a strong candidate as a feedstock for biofuel production. The major fact that it requires no special soil or climate conditions is a

significant agro-energy feature. With growth dimensions 3-5 m high and 1-5 m spread, this plant can be grown between other high value biomass; thus increasing the yield per acre land. In order to achieve economically viable conversion of *B. davidii* to bioethanol, all components of this biomass must be used. Many high value added chemicals were found to be present in *B. davidii*, such as flavonoids, triterpenes, and carotenoids. These compounds should be extracted prior to biomass processing for biofuels. Furthermore, achieving high recovery of hemicellulosic sugars would be necessary because hemicellulose accounts to about 40% of the total carbohydrate in *B. davidii*. Lignin also must be utilized not only for in-house fuel source but also for co-product applications. Lignin has been used as a polymer substitution, a precursor for biofuels, and a source for making carbon fiber. Since *B. davidii* has higher hemicellulose and lignin but lower cellulose contents than other lignocellulosic material, the importance of utilizing all biomass components is definitely more crucial for *B. davidii* than other species.

In conclusion, if one would envision the concept of biorefinery, in which all components of biomass are fully used to make a range of fuels, chemicals, materials, heat, and power, *B. davidii* plant may well be cultivated for value added chemicals, hemicellulose and cellulose will be converted to fermentable sugars, and lignin will be recovered for utilization in different co-product applications.

CHAPTER 10

RECOMMENDATIONS FOR FUTURE WORK

Several other studies might be conducted to further understand the effect of pretreatment technologies on *Buddleja davidii* as well as optimization of bioethanol production. Some particularly attractive options are as follows:

- To increase the recovery of ethanol free hemicellulosic sugars, mild acid extraction of wood could be performed prior to ethanol organosolv pretreatment. Achieving high recovery of such sugars would be necessary to achieve favorable overall process economics because hemicellulose accounts to about ~40% of the total carbohydrate in *B. davidii*. Xylanase should then be used to hydrolyze any oligomers remained. Microorganisms capable of fermenting C6 and C5 sugars should be used for efficient conversion of this bioresource to ethanol.
- Pore size and volume available in the pretreated biomass has been shown to have an effect on enzymatic hydrolysis. Such feature should be studied for ethanol organosolv pretreatment of *B. davidii*.
- Studying the changes in the ultrastructure, crystallinity and DP of cellulose during enzymatic hydrolysis could help understand the hydrolysis mechanism and the specific cellulose characteristics that affect the efficiency of the hydrolysis. CP/MAS ^{13}C NMR and GPC analyses would be performed on the cellulose at different time intervals.

APPENDIX A

GLOSSARY[5]

Bioenergy: The production, conversion, and use of material directly or indirectly produced by photosynthesis (including organic waste) to manufacture fuels and substitutes for petrochemical and other energy-intensive products.

Biofuels: Biomass converted to liquid or gaseous fuels such as ethanol, methanol, methane, and hydrogen.

Biomass: Any plant-derived organic matter. Biomass available for energy on a sustainable basis includes herbaceous and woody energy crops, agricultural food and feed crops, agricultural crop wastes and residues, wood wastes and residues, aquatic plants, and other waste materials including some municipal wastes. Biomass is a very heterogeneous and chemically complex renewable resource.

Bioethanol - Ethanol produced from biomass feedstocks. This includes ethanol produced from the fermentation of crops, such as corn, as well as cellulosic ethanol produced from woody plants or grasses.

Biorefinery - A facility that processes and converts biomass into value-added products. These products can range from biomaterials to fuels such as ethanol or important feedstocks for the production of chemicals and other materials. Biorefineries can be based on a number of processing platforms using mechanical, thermal, chemical, and biochemical processes.

Klason lignin (acid insoluble lignin): Lignin obtained from wood after the non-lignin components of the wood have been removed with a prescribed sulfuric acid treatment. A specific type of acid-insoluble lignin analysis.

Pretreatment. Physical and/or chemical treatments to make the biomass (cellulose, hemicelluloses, pectin and lignin) accessible to enzymatic hydrolysis. In this step, the hemicellulose fraction of the biomass is broken down into simple sugars. A chemical reaction called hydrolysis occurs when dilute sulfuric acid is mixed with the biomass feedstock. In this hydrolysis reaction, the complex chains of sugars that make up the hemicellulose are broken, releasing simple sugars. The complex hemicellulose sugars are converted to a mix of soluble five-carbon sugars, xylose and arabinose, and soluble six-carbon sugars, mannose and galactose. A small portion of the cellulose is also converted to glucose in this step.

[5] Information gathered from Oakridge National Renewable Laboratory, National Renewable Energy Laboratory, and U.S. Department of Energy websites.

APPENDIX B

COVER IMAGE

VOLUME 107, ISSUE 5, 2010

APPENDIX C

COPYRIGHT PERMISSIONS

C.1 Permission from Biofuels, Bioproducts and Biorefining

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Feb 02, 2011

This is a License Agreement between Bassem B Hallac ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2600870392198
License date	Feb 02, 2011
Licensed content publisher	John Wiley and Sons
Licensed content publication	Biofuels, Bioproducts and Biorefining
Licensed content title	Analyzing cellulose degree of polymerization and its relevancy to cellulosic ethanol
Licensed content author	Bassem B. Hallac, Arthur J. Ragauskas
Licensed content date	Jan 1, 2011
Start page	n/a
End page	n/a
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Order reference number	
Total	0.00 USD

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time athttp://myaccount.copyright.com)

Terms and Conditions

1. The materials you have requested permission to reproduce (the "Materials") are protected by copyright.

2. You are hereby granted a personal, non-exclusive, non-sublicensable, non-transferable, worldwide, limited license to reproduce the Materials for the purpose specified in the licensing process. This license is for a one-time use only with a maximum distribution equal to the number that you identified in the licensing process. Any form of republication granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before may be distributed thereafter). The Materials shall not be used in any other manner or for any other purpose. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher and on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Material. Any third party material is expressly excluded from this permission.

3. With respect to the Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Materials, or any of the rights granted to you hereunder to any other person.

4. The Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc or one of its related companies (WILEY) or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

5. NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. Any fee required for this permission shall be non-refundable after thirty (30) days from receipt.

13. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

14. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

15. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

16. This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

17. This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registred or certified mail, return receipt requested, at the last known address of such party. . BY CLICKING ON THE "I ACCEPT" BUTTON, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.

v1.4

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10924516.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

C.2 Permission from Trends in Plant Science

ELSEVIER LICENSE
TERMS AND CONDITIONS

Feb 02, 2011

This is a License Agreement between Bassem B Hallac ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Bassem B Hallac
Customer address	4801 Danube Ln. Durham, NC 27704
License number	2592650613620
License date	Jan 19, 2011
Licensed content publisher	Elsevier
Licensed content publication	Trends in Plant Science
Licensed content title	Lignins and lignocellulosics: a better control of synthesis for new and improved uses
Licensed content author	Alain M. Boudet, Shinya Kajita, Jacqueline Grima-Pettenati, Deborah Goffner
Licensed content date	December 2003
Licensed content volume number	8
Licensed content issue number	12
Number of pages	6
Start Page	576
End Page	581
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Fundamental Understanding of the Biochemical Conversion of Buddleja davidii to Bioethanol
Expected completion date	Apr 2011
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
Value added tax 0.0%	0.0 USD / 0.0 GBP

Total 0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed

immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing athttp://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and
he permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx , As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com

All content posted to the web site must maintain the copyright information line on the bottom of each image.
You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing
at http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10916346.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2592650613620 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

C.3 Permission from Nature Reviews Genetics

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Feb 02, 2011

This is a License Agreement between Bassem B Hallac ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2600860625589
License date	Feb 02, 2011
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Genetics
Licensed content title	Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol
Licensed content author	Mariam B. Sticklen
Licensed content date	Jun 1, 2008
Volume number	9
Issue number	6
Type of Use	reuse in a thesis/dissertation
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1 C
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Fundamental Understanding of the Biochemical Conversion of Buddleja davidii to Bioethanol
Expected completion date	Apr 2011
Estimated size (number of pages)	200
Total	0.00 USD

Terms and Conditions

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, **and for no other use**, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the

material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

   Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run).

   NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME](reference citation), copyright (year of publication)

   For AOP papers, the credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME](reference citation), copyright (year of publication)

7. Translations of up to a whole article do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME](reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

C.4 Permission from Bioresource Technology

ELSEVIER LICENSE
TERMS AND CONDITIONS

Feb 02, 2011

This is a License Agreement between Bassem B Hallac ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Bassem B Hallac
Customer address	4801 Danube Ln. Durham, NC 27704
License number	2600850786685
License date	Feb 02, 2011
Licensed content publisher	Elsevier
Licensed content publication	Bioresource Technology
Licensed content title	Features of promising technologies for pretreatment of lignocellulosic biomass
Licensed content author	Nathan Mosier, Charles Wyman, Bruce Dale, Richard Elander, Y.Y. Lee, Mark Holtzapple, Michael Ladisch
Licensed content date	April 2005
Licensed content volume number	96
Licensed content issue number	6
Number of pages	14
Start Page	673
End Page	686
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Fundamental Understanding of the Biochemical Conversion of Buddleja davidii to Bioethanol
Expected completion date	Apr 2011
Estimated size (number of pages)	200
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD

Value added tax 0.0%	0.00 USD / GBP
Total	0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment

terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request,

other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com , and
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and
he permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx , As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.
Central Storage: This license does not include permission for a scanned version of the

material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com

All content posted to the web site must maintain the copyright information line on the bottom of each image. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

C.5 Permission from Journal of Agricultural and Food Chemistry

AMERICAN CHEMICAL SOCIETY LICENSE TERMS AND CONDITIONS

Feb 02, 2011

This is a License Agreement between Bassem B Hallac ("You") and American Chemical Society ("American Chemical Society") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by American Chemical Society, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2504281122252
License Date	Sep 08, 2010
Licensed content publisher	American Chemical Society
Licensed content publication	Journal of Agricultural and Food Chemistry
Licensed content title	Biomass Characterization of Buddleja davidii: A Potential Feedstock for Biofuel Production
Licensed content author	Bassem B. Hallac et al.
Licensed content date	Feb 1, 2009
Volume number	57
Issue number	4
Type of Use	Thesis/Dissertation
Requestor type	Not specified
Format	Print
Portion	Full article
Author of this ACS article	Yes
Order reference number	
Title of the thesis / dissertation	Fundamental Understanding of the Biochemical Conversion of Buddleja davidii to Bioethanol
Expected completion date	Apr 2011
Estimated size(pages)	200
Billing Type	Invoice
Billing Address	4801 Danube Ln. Apt 723 Durham, NC 27704 United States
Customer reference info	
Total	0.00 USD
Terms and Conditions	

Thesis/Dissertation

ACS / RIGHTSLINK TERMS & CONDITIONS
THESIS/DISSERTATION

INTRODUCTION
The publisher for this copyrighted material is the American Chemical Society. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

LIMITED LICENSE
Publisher hereby grants to you a non-exclusive license to use this material. Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process; any form of republication must be completed within 60 days from the date hereof (although copies prepared before then may be distributed thereafter).

GEOGRAPHIC RIGHTS: SCOPE
Licenses may be exercised anywhere in the world.

RESERVATION OF RIGHTS
Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

PORTION RIGHTS STATEMENT: DISCLAIMER
If you seek to reuse a portion from an ACS publication, it is your responsibility to examine each portion as published to determine whether a credit to, or copyright notice of, a third party owner was published adjacent to the item. You may only obtain permission via Rightslink to use material owned by ACS. Permission to use any material published in an ACS publication, journal, or article which is reprinted with permission of a third party must be obtained from the third party owner. ACS disclaims any responsibility for any use you make of items owned by third parties without their permission.

REVOCATION
The American Chemical Society reserves the right to revoke a license for any reason, including but not limited to advertising and promotional uses of ACS content, third party usage, and incorrect figure source attribution.

LICENSE CONTINGENT ON PAYMENT
While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (by CCC) as provided in CCC's Billing and Payment

terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

COPYRIGHT NOTICE: DISCLAIMER
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Reprinted (“Adapted” or “in part”) with permission from REFERENCE CITATION. Copyright YEAR American Chemical Society."

WARRANTIES: NONE
Publisher makes no representations or warranties with respect to the licensed material.

INDEMNITY
You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

NO TRANSFER OF LICENSE
This license is personal to you or your publisher and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

NO AMENDMENT EXCEPT IN WRITING
This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

OBJECTION TO CONTRARY TERMS
Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

JURISDICTION
This license transaction shall be governed by and construed in accordance with the laws of the District of Columbia. You hereby agree to submit to the jurisdiction of the courts located in the District of Columbia for purposes of resolving any disputes that may arise

in connection with this licensing transaction.

THESES/DISSERTATION TERMS

Publishing implications of electronic publication of theses and dissertation material

Students and their mentors should be aware that posting of theses and dissertation material on the Web prior to submission of material from that thesis or dissertation to an ACS journal mayaffect publication in that journal. Whether Web posting is considered prior publication may be evaluated on a case-by-case basis by the journal's editor. If an ACS journal editor considers Web posting to be "prior publication", the paper will not be accepted for publication in that journal. If you intend to submit your unpublished paper to ACS for publication, check with the appropriate editor prior to posting your manuscript electronically.

If your paper has already been published by ACS and you want to include the text or portions of the text in your thesis/dissertation in **print or microfilm formats**, please print the ACS copyright credit line on the first page of your article: "Reproduced (or 'Reproduced in part') with permission from [FULL REFERENCE CITATION.] Copyright [YEAR] American Chemical Society." Include appropriate information.

Submission to a Dissertation Distributor: If you plan to submit your thesis to UMI or to another dissertation distributor, you should not include the unpublished ACS paper in your thesis if the thesis will be disseminated electronically, until ACS has published your paper. After publication of the paper by ACS, you may release the entire thesis (**not the individual ACSarticle by itself**) for electronic dissemination through the distributor; ACS's copyright credit line should be printed on the first page of the ACS paper.

Use on an Intranet: The inclusion of your ACS unpublished or published manuscript is permitted in your thesis in print and microfilm formats. If ACS has published your paper you may include the manuscript in your thesis on an intranet that is not publicly available. Your ACS article cannot be posted electronically on a publicly available medium (i.e. one that is not password protected), such as but not limited to, electronic archives, Internet, library server, etc. The only material from your paper that can be posted on a public electronic medium is the article abstract, figures, and tables, and you may link to the article's DOI or post the article's author-directed URL link provided by ACS. This paragraph does not pertain to the dissertation distributor paragraph above.

Other conditions:

v1.1

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10845446.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001

Dear Bassem Hallac,

This is in reference to your message below.

Permission is granted for you to include the specified ACS journal article in your thesis in electronic format.

Hope this information is helpful.

C. Arleen Courtney
Copyright Associate
Copyright, Permissions, & Licensing
Office of Secretary & General Counsel
American Chemical Society
1155 Sixteenth Street, NW
Washington, DC 20036
Email. a_courtney@acs.org / Dept. Email. copyright@acs.org
Tel. 202-872-4368 / Fax. 202-776-8112

-----Original Message-----
From: bhallac3@gatech.edu [mailto:bhallac3@gatech.edu]
Sent: Wednesday, September 08, 2010 11:43 AM
To: Copyright
Subject: Re: Permission

Thanks.

I am the author of the following article.

Bassem B. Hallac, Poulomi Sannigrahi, Yunqiao Pu, Michael Ray, Richard J. Murphy, and Arthur J. Ragauskas. "Biomass Characterization of Buddleja davidii: A Potential Feedstock for Biofuel Production". Journal of Agricultural and Food Chemistry, 57 (4), 1275-1281, 2009

I went through Rightslink to obtain a permission to use this article in my thesis both in an electronic and print format, but the system did not allow me to obtain the electronic one.

I contacted the RightsLink office, they couldn't help me and asked to me to contact the ACS copyright office.

My thesis will be available in an electronic and print format.

Thanks

Bassem Hallac
Graduate Student
School of Chemistry and Biochemistry
Institute of Paper Science and Technology
Georgia Institute of Technology
Atlanta, GA 30332

----- Original Message -----
From: "Copyright" <Copyright@acs.org>
To: "Bassem B Hallac" <bhallac3@gatech.edu>
Sent: Wednesday, September 8, 2010 9:19:24 AM GMT -05:00 US/Canada Eastern
Subject: RE: Permission

Dear Bassem Hallac,

This is in reference to your message below.

In case you did not receive our out-of-office message when you sent us your request, our office is no longer accepting permission requests or forms (either by email, fax, or mail) for ACS journal material (with the exception of certain situations listed below). In addition, our office no longer sends letters to students regarding their theses/dissertations.

Please use the Rightslink permission system to obtain permission to use the ACS journal material. A quick way to get into the Rightslink system is to go to <pubs.acs.org>:

Click on Journals A-Z and look at the list of journal titles and select the journal you want to use.

At the top right hand part of that page, click on citation and enter the volume number and number of the first page of the article. The abstract for that article will appear on your screen.

Then click on the Rights & Permissions box located on the right side of the article abstract. That will take you into the Rightslink permission system.

Set up your Rightslink account. In the "portion to the used section" of Rightslink, click on "table/figure/micrograph" and enter the number of figures (not the figure numbers)

you want to use from the article or from the table of contents whether you are using them verbatim, adapting them, or using them in part. If you are using text, click on the amount of text you want to use. Click on the type of publication in which your manuscript will appear in the section that asks you where will the ACS material appear. If you do not see a selection exactly like your type of use, select the closest selection to your type of use. Our permission is not needed for the use of schemes or structures.

If you are using material from more than one ACS journal, repeat the above process. You don't need to set up your account again.

Once your order has been processed, you will be able to check to see if a fee will be charged by looking at the Quick Price Page. If you decide not to use the material, you can cancel your order.

If you continue with your order, you will receive a link to the Terms and Conditions that you can print out and send to your publisher/university showing that you obtained permission. The appropriate credit line will be included in the Terms and Conditions.

Most major publishers are now using the Rightslink permission system so after you set up your Rightslink account, you can use that account to obtain permission for other publishers' material if they use Rightslink. Suggestion: Before sending permission request forms to any publisher, check their Web sites to see how they want you to apply for permission.

How to Handle Problems With Your Rightslink Order
Please be aware that under certain conditions you may receive from Rightslink the following message: "The Copyright status of this article must be verified". You should review the three scenarios below to help you determine why your order prompted the message.

Scenario #1: The IT system that ACS uses to support the Rightslink permission system does not always recognize articles; and therefore requesters receive the above message.

Scenario #2: Requesters who want to use material that appeared in articles that are in the Public Domain (all of the authors on the paper worked for the U.S. Government when the paper was written) do not need our permission if the notice on the article states: "Not subject to U.S. Copyright". Before you place your Rightslink order, check the copyright notice that appears with the abstract or on the first page of the article.

Scenario #3: All of the authors on a requested article worked for the Governments of Australia, Canada, or the UK. Requesters need to write to the authors of the article for permission because the authors' countries own copyright to those articles. For these articles, a "Crown" copyright notice appears with the abstract. Before you place your Rightlink order, check the notice that appears with the abstract or on the first page of the article.

If you receive "The Copyright status of this article must be verified" notice and you checked the copyright notice and it states "Copyright YEAR American Chemical Society" and neither Scenario #2 or 3 apply, then send to copyright@acs.org a copy of the message you received and a permission request that includes a complete reference citation, the amount of material you want to use from the article (such as the specific ACS figure numbers), and where you plan to reuse the ACS material.

If there is some other problem with your Rightslink order, please contact the customer service at the Copyright Clearance Center. They can be reached by email at <customercare@copyright.com> or by phone at +1 877-622-5543 or +1-978-646-2777. They can assist you with your problem.

Hope this information is helpful.

C. Arleen Courtney
Copyright Associate
Copyright, Permissions, & Licensing
Office of Secretary & General Counsel
American Chemical Society
1155 Sixteenth Street, NW
Washington, DC 20036
Email. a_courtney@acs.org / Dept. Email. copyright@acs.org
Tel. 202-872-4368 / Fax. 202-776-8112

-----Original Message-----
From: Hallac, Bassem B [mailto:bhallac3@gatech.edu]
Sent: Tuesday, September 07, 2010 11:11 PM
To: Copyright
Subject: Permission

Hi,

I am preparing my Ph.D thesis for publication at Georgia Tech.

I would need your permission to use the following material:

Bassem B. Hallac, Poulomi Sannigrahi, Yunqiao Pu, Michael Ray, Richard J. Murphy, and Arthur J. Ragauskas. "Biomass Characterization of Buddleja davidii: A Potential Feedstock for Biofuel Production". Journal of Agricultural and Food Chemistry, 57 (4), 1275-1281, 2009

Is there anything specific I need to mention in my dissertation?
Thanks

C.6 Permission from Industrial & Engineering Chemistry Research

AMERICAN CHEMICAL SOCIETY LICENSE
TERMS AND CONDITIONS

Feb 02, 2011

This is a License Agreement between Bassem B Hallac ("You") and American Chemical Society ("American Chemical Society") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by American Chemical Society, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2558390417362
License Date	Nov 29, 2010
Licensed content publisher	American Chemical Society
Licensed content publication	Industrial & Engineering Chemistry Research
Licensed content title	Effect of Ethanol Organosolv Pretreatment on Enzymatic Hydrolysis of Buddleja davidii Stem Biomass
Licensed content author	Bassem B. Hallac et al.
Licensed content date	Feb 1, 2010
Volume number	49
Issue number	4
Type of Use	Thesis/Dissertation
Requestor type	Not specified
Format	Print
Portion	Full article
Author of this ACS article	Yes
Order reference number	
Title of the thesis / dissertation	Fundamental Understanding of the Biochemical Conversion of Buddleja davidii to Bioethanol
Expected completion date	Apr 2011
Estimated size(pages)	200
Billing Type	Invoice
Billing Address	4801 Danube Ln. Apt 723 Durham, NC 27704 United States
Customer reference info	
Total	0.00 USD
Terms and Conditions	

Thesis/Dissertation

ACS / RIGHTSLINK TERMS & CONDITIONS

THESIS/DISSERTATION

INTRODUCTION
The publisher for this copyrighted material is the American Chemical Society. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

LIMITED LICENSE
Publisher hereby grants to you a non-exclusive license to use this material. Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process; any form of republication must be completed within 60 days from the date hereof (although copies prepared before then may be distributed thereafter).

GEOGRAPHIC RIGHTS: SCOPE
Licenses may be exercised anywhere in the world.

RESERVATION OF RIGHTS
Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

PORTION RIGHTS STATEMENT: DISCLAIMER
If you seek to reuse a portion from an ACS publication, it is your responsibility to examine each portion as published to determine whether a credit to, or copyright notice of, a third party owner was published adjacent to the item. You may only obtain permission via Rightslink to use material owned by ACS. Permission to use any material published in an ACS publication, journal, or article which is reprinted with permission of a third party must be obtained from the third party owner. ACS disclaims any responsibility for any use you make of items owned by third parties without their permission.

REVOCATION
The American Chemical Society reserves the right to revoke a license for any reason, including but not limited to advertising and promotional uses of ACS content, third party usage, and incorrect figure source attribution.

LICENSE CONTINGENT ON PAYMENT
While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of

CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

COPYRIGHT NOTICE: DISCLAIMER
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Reprinted (“Adapted” or “in part”) with permission from REFERENCE CITATION. Copyright YEAR American Chemical Society."

WARRANTIES: NONE
Publisher makes no representations or warranties with respect to the licensed material.

INDEMNITY
You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

NO TRANSFER OF LICENSE
This license is personal to you or your publisher and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

NO AMENDMENT EXCEPT IN WRITING
This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

OBJECTION TO CONTRARY TERMS
Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

JURISDICTION
This license transaction shall be governed by and construed in accordance with the laws of the District of Columbia. You hereby agree to submit to the jurisdiction of the courts located in the District of Columbia for purposes of resolving any disputes that may arise in connection with this licensing transaction.

THESES/DISSERTATION TERMS
Publishing implications of electronic publication of theses and dissertation material

Students and their mentors should be aware that posting of theses and dissertation material on the Web prior to submission of material from that thesis or dissertation to an ACS journal mayaffect publication in that journal. Whether Web posting is considered prior publication may be evaluated on a case-by-case basis by the journal's editor. If an ACS journal editor considers Web posting to be "prior publication", the paper will not be accepted for publication in that journal. If you intend to submit your unpublished paper to ACS for publication, check with the appropriate editor prior to posting your manuscript electronically.

If your paper has already been published by ACS and you want to include the text or portions of the text in your thesis/dissertation in **print or microfilm formats**, please print the ACS copyright credit line on the first page of your article: "Reproduced (or 'Reproduced in part') with permission from [FULL REFERENCE CITATION.] Copyright [YEAR] American Chemical Society." Include appropriate information.

Submission to a Dissertation Distributor: If you plan to submit your thesis to UMI or to another dissertation distributor, you should not include the unpublished ACS paper in your thesis if the thesis will be disseminated electronically, until ACS has published your paper. After publication of the paper by ACS, you may release the entire thesis (**not the individual ACSarticle by itself**) for electronic dissemination through the distributor; ACS's copyright credit line should be printed on the first page of the ACS paper.

Use on an Intranet: The inclusion of your ACS unpublished or published manuscript is permitted in your thesis in print and microfilm formats. If ACS has published your paper you may include the manuscript in your thesis on an intranet that is not publicly available. Your ACS article cannot be posted electronically on a publicly available medium (i.e. one that is not password protected), such as but not limited to, electronic archives, Internet, library server, etc. The only material from your paper that can be posted on a public electronic medium is the article abstract, figures, and tables, and you may link to the article's DOI or post the article's author-directed URL link provided by ACS. This paragraph does not pertain to the dissertation distributor paragraph above.

Other conditions:

v1.1

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10891691.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2558390417362 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Dear Bassem Hallac,

This is in reference to your message below.

If you are a student and your university mandates that you obtain both print and electronic rights to include the ACS material in your thesis/dissertation, even though the Terms & Conditions allow print rights only, you also have electronic rights as well so print out a copy of this message and give it to your Graduate School.

Hope this information is helpful.

C. Arleen Courtney
Copyright Associate
Copyright, Permissions, & Licensing
Office of Secretary & General Counsel
American Chemical Society
1155 Sixteenth Street, NW
Washington, DC 20036
Email. a_courtney@acs.org / Dept. Email. copyright@acs.org
Tel. 202-872-4368 / Fax. 202-776-8112

-----Original Message-----
From: Hallac, Bassem B [mailto:bhallac3@gatech.edu]
Sent: Monday, November 29, 2010 4:39 PM
To: Copyright
Subject: Copyright Permission

Hi,

I am the author of the following article.

Bassem B. Hallac, Poulomi Sannigrahi, Yunqiao Pu, Michael Ray, Richard J. Murphy, and Arthur J. Ragauskas. “Effect of Ethanol Organosolv Pretreatment on Enzymatic Hydrolysis of Buddleja davidii Stem Biomass”. Industrial & Engineering Chemistry Research, 49 (4), 1467-1472, 2010

I went through Rightslink to obtain a permission to use this article in my thesis both in an electronic and print format, but the system did not allow me to obtain both. I had to chose one option. So now I have the copyright for the print format.

I contacted the RightsLink office, they couldn't help me and asked to me to contact the ACS copyright office.

My thesis will be available in an electronic and print format.

Can I be granted the copyright for the electronic format for this article?

Thanks

Bassem Hallac
Graduate Student
School of Chemistry and Biochemistry
Institute of Paper Science and Technology Georgia Institute of Technology Atlanta, GA 30332

C.7 Permission from Energy & Fuels

AMERICAN CHEMICAL SOCIETY LICENSE
TERMS AND CONDITIONS
Feb 02, 2011

This is a License Agreement between Bassem B Hallac ("You") and American Chemical Society ("American Chemical Society") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by American Chemical Society, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2559081378537
License Date	Nov 30, 2010
Licensed content publisher	American Chemical Society
Licensed content publication	Energy & Fuels
Licensed content title	Chemical Transformations of Buddleja davidii Lignin during Ethanol Organosolv Pretreatment
Licensed content author	Bassem B. Hallac et al.
Licensed content date	Apr 1, 2010
Volume number	24
Issue number	4
Type of Use	Thesis/Dissertation
Requestor type	Not specified
Format	Print
Portion	Full article
Author of this ACS article	Yes
Order reference number	
Title of the thesis / dissertation	Fundamental Understanding of the Biochemical Conversion of Buddleja davidii to Bioethanol
Expected completion date	Apr 2011
Estimated size(pages)	200
Billing Type	Invoice
Billing Address	4801 Danube Ln. Apt 723 Durham, NC 27704 United States
Customer reference info	
Total	0.00 USD
Terms and Conditions	

Thesis/Dissertation

ACS / RIGHTSLINK TERMS & CONDITIONS
THESIS/DISSERTATION

INTRODUCTION
The publisher for this copyrighted material is the American Chemical Society. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

LIMITED LICENSE
Publisher hereby grants to you a non-exclusive license to use this material. Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process; any form of republication must be completed within 60 days from the date hereof (although copies prepared before then may be distributed thereafter).

GEOGRAPHIC RIGHTS: SCOPE
Licenses may be exercised anywhere in the world.

RESERVATION OF RIGHTS
Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

PORTION RIGHTS STATEMENT: DISCLAIMER
If you seek to reuse a portion from an ACS publication, it is your responsibility to examine each portion as published to determine whether a credit to, or copyright notice of, a third party owner was published adjacent to the item. You may only obtain permission via Rightslink to use material owned by ACS. Permission to use any material published in an ACS publication, journal, or article which is reprinted with permission of a third party must be obtained from the third party owner. ACS disclaims any responsibility for any use you make of items owned by third parties without their permission.

REVOCATION
The American Chemical Society reserves the right to revoke a license for any reason, including but not limited to advertising and promotional uses of ACS content, third party usage, and incorrect figure source attribution.

LICENSE CONTINGENT ON PAYMENT
While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (by CCC) as provided in CCC's Billing and Payment terms

and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

COPYRIGHT NOTICE: DISCLAIMER
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Reprinted (“Adapted” or “in part”) with permission from REFERENCE CITATION. Copyright YEAR American Chemical Society."

WARRANTIES: NONE
Publisher makes no representations or warranties with respect to the licensed material.

INDEMNITY
You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

NO TRANSFER OF LICENSE
This license is personal to you or your publisher and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

NO AMENDMENT EXCEPT IN WRITING
This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

OBJECTION TO CONTRARY TERMS
Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

JURISDICTION
This license transaction shall be governed by and construed in accordance with the laws of the District of Columbia. You hereby agree to submit to the jurisdiction of the courts located in the District of Columbia for purposes of resolving any disputes that may arise

in connection with this licensing transaction.

THESES/DISSERTATION TERMS

Publishing implications of electronic publication of theses and dissertation material

Students and their mentors should be aware that posting of theses and dissertation material on the Web prior to submission of material from that thesis or dissertation to an ACS journal mayaffect publication in that journal. Whether Web posting is considered prior publication may be evaluated on a case-by-case basis by the journal's editor. If an ACS journal editor considers Web posting to be "prior publication", the paper will not be accepted for publication in that journal. If you intend to submit your unpublished paper to ACS for publication, check with the appropriate editor prior to posting your manuscript electronically.

If your paper has already been published by ACS and you want to include the text or portions of the text in your thesis/dissertation in **print or microfilm formats**, please print the ACS copyright credit line on the first page of your article: "Reproduced (or 'Reproduced in part') with permission from [FULL REFERENCE CITATION.] Copyright [YEAR] American Chemical Society." Include appropriate information.

Submission to a Dissertation Distributor: If you plan to submit your thesis to UMI or to another dissertation distributor, you should not include the unpublished ACS paper in your thesis if the thesis will be disseminated electronically, until ACS has published your paper. After publication of the paper by ACS, you may release the entire thesis (**not the individual ACSarticle by itself**) for electronic dissemination through the distributor; ACS's copyright credit line should be printed on the first page of the ACS paper.

Use on an Intranet: The inclusion of your ACS unpublished or published manuscript is permitted in your thesis in print and microfilm formats. If ACS has published your paper you may include the manuscript in your thesis on an intranet that is not publicly available. Your ACS article cannot be posted electronically on a publicly available medium (i.e. one that is not password protected), such as but not limited to, electronic archives, Internet, library server, etc. The only material from your paper that can be posted on a public electronic medium is the article abstract, figures, and tables, and you may link to the article's DOI or post the article's author-directed URL link provided by ACS. This paragraph does not pertain to the dissertation distributor paragraph above.

Other conditions:

v1.1

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10892350.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001

C.8 Permission from Biotechnology and Bioengineering

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Feb 02, 2011

This is a License Agreement between Bassem B Hallac ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2560381112188
License date	Dec 01, 2010
Licensed content publisher	John Wiley and Sons
Licensed content publication	Biotechnology & Bioengineering
Licensed content title	Correlation between anatomical characteristics of ethanol organosolv pretreated Buddleja davidii and its enzymatic conversion to glucose
Licensed content author	Bassem B. Hallac,Michael Ray,Richard J. Murphy,Arthur J. Ragauskas
Licensed content date	Dec 1, 2010
Start page	795
End page	801
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Order reference number	
Total	0.00 USD
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one if its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

1. The materials you have requested permission to reproduce (the "Materials") are protected by copyright.

2. You are hereby granted a personal, non-exclusive, non-sublicensable, non-transferable, worldwide, limited license to reproduce the Materials for the purpose specified in the licensing process. This license is for a one-time use only with a maximum distribution equal to the number that you identified in the licensing process. Any form of republication granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before may be distributed thereafter). Any electronic posting of the Materials is limited to one year from the date permission is granted and is on the condition that a link is placed to the journal homepage on Wiley's online journals publication platform at www.interscience.wiley.com. The Materials shall not be used in any other manner or for any other purpose. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher and on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Material. Any third party material is expressly excluded from this permission.

3. With respect to the Materials, all rights are reserved. No part of the Materials may be copied, modified, adapted, translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Materials, or any of the rights granted to you hereunder to any other person.

4. The Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc or one of its related companies (WILEY) or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

5. WILEY DOES NOT MAKE ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in a writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

13. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

14. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

15. This Agreement shall be governed by and construed in accordance with the laws of England and you agree to submit to the exclusive jurisdiction of the English courts.

16. Other Terms and Conditions:

BY CLICKING ON THE "I ACCEPT" BUTTON, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET FORTH IN THIS AGREEMENT.

V1.2

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10892958.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that

time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2560381112188 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Mar 25, 2011

This is a License Agreement between Bassem B Hallac ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2636101348646
License date	Mar 25, 2011
Licensed content publisher	John Wiley and Sons
Licensed content publication	Biotechnology & Bioengineering
Licensed content title	Correlation between anatomical characteristics of ethanol organosolv pretreated Buddleja davidii and its enzymatic conversion to glucose
Licensed content author	Bassem B. Hallac,Michael Ray,Richard J. Murphy,Arthur J. Ragauskas
Licensed content date	Dec 1, 2010
Start page	795
End page	801
Type of use	I don't see my intended use
Special requirements	To use the cover image on this issue in my thesis, both in a print and electronic format.
Order reference number	
Total	0.00 USD
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time athttp://myaccount.copyright.com)

Terms and Conditions

1. The materials you have requested permission to reproduce (the "Materials") are protected by copyright.

2. You are hereby granted a personal, non-exclusive, non-sublicensable, non-transferable, worldwide, limited license to reproduce the Materials for the purpose specified in the licensing process. This license is for a one-time use only with a maximum distribution equal to the number that you identified in the licensing process. Any form of republication granted by this licence must be completed within two years of the date of the grant of this licence (although copies prepared before may be distributed thereafter). The Materials shall not be used in any other manner or for any other purpose. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher and on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Material. Any third party material is expressly excluded from this permission.

3. With respect to the Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Materials without the prior permission of the respective copyright owner. You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Materials, or any of the rights granted to you hereunder to any other person.

4. The Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc or one of its related companies (WILEY) or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto.

5. NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED BY YOU.

6. WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

7. You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

8. IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

9. Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

10. The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

11. This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.

12. Any fee required for this permission shall be non-refundable after thirty (30) days from receipt.

13. These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.

14. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

15. WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

16. This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

17. This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registred or certified mail, return receipt requested, at the last known address of such party. . BY CLICKING ON THE "I ACCEPT" BUTTON, YOU ACKNOWLEDGE THAT YOU HAVE READ AND FULLY UNDERSTAND EACH OF THE SECTIONS OF AND PROVISIONS SET FORTH IN THIS AGREEMENT AND THAT YOU ARE IN AGREEMENT WITH AND ARE WILLING TO ACCEPT ALL OF YOUR OBLIGATIONS AS SET

FORTH IN THIS AGREEMENT.

v1.4

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10957290.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact Rightslink Customer Support:customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

REFERENCES

[1] Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T. The path forward for biofuels and biomaterials. Science 2006;311 (5760):484-489.

[2] Pu Y, Zhang D, Singh PM, Ragauskas AJ. The new forestry biofuels sector. Biofuel Bioprod Bior 2007;2 (1):58-73.

[3] Ragauskas AJ, Nagy Mate, Kim DH, Eckert CA, Hallett JP, Liotta CL. From wood to fuels: Integrating biofuels and pulp production. Ind Biotechnol 2006;2 (1):55-65.

[4] Sannigrahi P, Pu Y, Ragauskas A. Cellulosic biorefineries—unleashing lignin opportunities. Curr Opin Environ Sustainability 2010;2 (5-6):383-393 .

[5] David K, Ragauskas AJ. Switchgrass as an energy crop for biofuel production: A review of its ligno-cellulosic chemical properties. Energy Environ Sci 2010;3 1182–1190.

[6] Sannigrahi P, Ragauskas AJ, Tuskan GA. Poplar as a feedstock for biofuels: A review of compositional characteristics. Biofuel Bioprod Bior 2010;4 (2):209-226.

[7] Galbe M, Zacchi G. Pretreatment of lignocellulosic materials for efficient bioethanol production. Adv Biochem Eng Biot 2007;108 41-65.

[8] Yang B, Wyman CE. Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuel Bioprod Bior 2008;2 (1):26-40.

[9] Jeffries TW, Jin Y. Metabolic engineering for improved fermentation of pentoses by yeasts. Appl Microbiol Biot 2004;63 (5):495–509.

[10] Chandra RP, Bura R, Mabee WE, Berlin A, Pan X, Saddler JN. Substrate pretreatment: They key to effective enzymatic hydrolysis of lignocellulosics? Adv Biochem Eng Biot 2007;108 67-93.

[11] Samuel R, Pu Y, Raman B, Ragauskas AJ. Structural characterization and comparison of switchgrass lignin before and after dilute acid pretreatment. Appl Biochem Biotech 2010;162 (1):62-74.

[12] Zhang YP. Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. J Ind Microbiol Biot 2008;35 (5):367-375.

[13] Lange JP. Lignocellulose conversion: an introduction to chemistry, process and economics. Biofuel Bioprod Bior 2007;1 (1):39-48.

[14] U.S. Energy Information Administration. http://www.eia.doe.gov/emeu/international/contents.html (December 2010).

[15] Rubin EM. Genomics of cellulosic biofuels. Nature 2008;454 841-845.

[16] Himmel ME, Ding SY, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD. Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Science 2007;315 (5813):804-807.

[17] U.S. Department of Energy/Energy Efficiency and Renewable Energy, http://www1.eere.energy.gov/biomass/environmental.html (December, 2010).

[18] Gomez LD, Steele-King CG, McQueen-Mason J. Sustainable liquid biofuels from biomass: the writing's on the walls. New Phytol 2008;178 (2):473-485.

[19] Wang M. Updated energy and greenhouse gas emission results of fuel ethanol. In: The 15th international symposium on alcohol fules, San Diego, CA, 2005.

[20] Kumar P, Barrett DM, Delwiche MJ, P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 2009;48 (8):3713-3729.

[21] Chin S. Poplar as a feedstock for biofuel production. Imperial College London, MSci thesis 2009.

[22] Kamm B, Kamm M. Principles of biorefineries. Appl Microbiol Biot 2004;64 (2):137-145.

[23] Somerville C, Youngs H, Taylor C, Davis SC, Long SP. Feedstocks for lignocellulosic biofuels. Science 2010;329 (5993):790-792.

[24] Findley DA, Keever GJ, Chappelka AH, Eakes DJ, Gilliam CH. Differential response of buddleia (*Buddleia davidii* Franch.) to ozone. Environ Pollut 1997;98 (1):105-111.

[25] Bonciani G. Exploration of *Buddleja davidii* (the butterfly bush) as a novel bioenergy crop. Imperial College London, MRes thesis 2009.

[26] Starr F, Starr K, Loope L. *Buddleia davidii*: Butterfly Bush: Buddleiaceae. United States Geological Survey (Biological Resources Division): Hawaii, 2008; available at http://www.hear.org/pier/pdf/pohreports/buddleia_davidii.pdf.

[27] U.S. Department of Energy: Natural Resources Conservation Service, http://plants.usda.gov/java/profile?symbol=BUDA2, (January, 2011).

[28] Humphries RN, Guarino L. The effect of water stress on mortality of *Betula pendula* Roth. and *Buddleia davidii* Franch. seedlings. Plant Soil 1982;64 (2):273-276.

[29] Esler AE. The naturalisation of plants in urban Auckland, New Zealand. 5. Success of the alien species. New Zeal J Bot 1988;26 (4):565-584.

[30] Genome programs of the U.S. department of energy office of sceince. http://genomics.energy.gov/gallery/brc/detail.np/detail-06.html (December 2010).

[31] Houghton PJ, Hylands PJ, Mensah AY, Hensel A, Deters AM. In vitro tests and ethnopharmacological investigations: Wound healing as an example. J Ethnopharmacol 2005;100 (1-2):100-107.

[32] Houghton PJ, Mensaha AY, Iessa N, Honga LY. Terpenoids in *Buddleja*: relevance to chemosystematics, chemical ecology and biological activity. Phytochemistry 2003;64 (2):385-393.

[33] Mensah AY, Sampson J, Houghton PJ, Hylands PJ, Westbrook J, Dunn M, Hughes MA, Cherry GW. Effects of *Buddleja globosa* leaf and its constituents relevant to wound healing. J Ethnopharmacol 2001;77 (2-3):219–226.

[34] Sadyrbekov DT, Atazhanova GA, Kulyyasov AT, Raldugin VA, Gatilov YV, Shakirov MM, Edil'baeva TT, Turdybekov KM, Adekenov SM. Buddledin C from *Pulicaria prostrata* and selective synthesis of its epoxy derivative. Chem Nat Compd 2006;42 (1):41-45.

[35] Fengel D, Wegener G. Wood. Chemistry, ultrastructure, reactions. Walter de Gruyter: Berlin, 1984. pp. 613.

[36] Boudet AM, Kajita S, Grima-Pettenati J, Goffner D. Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends in Plant Science 2003;8 (12):576-581.

[37] Saha BC. Hemicellulose bioconversion. J Ind Microbiol Biot 2003;30 (5):279-291.

[38] Sticklen MB. Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nat Rev Genet 2008;9 433-443.

[39] Shafizadeh F, McGinnis GD. Morphology and biogenesis of cellulose and plant cell-walls. In: Advances in In: Advances in carbohydrate chemistry and biochemistry. Tipson RS, editors. Academic Press: New York, 1971. pp. 297-351.

[40] Sjöström E. Wood chemistry: fundamentals and applications. Academic Press: San Diego, California, 1981. pp. 223.

[41] Sarkanen KV, Ludwig CH. Lignin: occurrence, formation, structure and reactions. Wiley Interscience: New York, 1971. pp. 916.

[42] Nagy M. Biofuels from lignin and novel biodiesel analysis. Georgia Institute of Technology, Ph.D. thesis 2009.

[43] U.S. Department of Agriculture: Agricultural Research Service, http://ars.usda.gov/Services/docs.htm?docid=10443, (February 2011).

[44] Chakar FS, Ragauskas AJ. Review of current and future softwood kraft lignin process chemistry. Ind Crop Prod 2004;20 (2):131-141.

[45] Chakar FS. Fundamental delignification chemistry of laccase-mediator systems on high- lignin-content kraft pulps. Institute of Paper Science and Technology, Ph.D. thesis 2000.

[46] Boerjan W, Ralph J, Baucher M. Lignin biosynthesis. Annu Rev Plant Biol 2003;54 519–546.

[47] Capanema EA, Balakshin MY, Kadla JF. A comprehensive approach for quantitative lignin characterization by NMR spectroscopy. J Agr Food Chem 2004;52 (7):1850-1860.

[48] Capanema EA, Balakshin MY, Kadla JF. Quantitative characterization of a hardwood milled wood lignin by nuclear magnetic resonance spectroscopy. J Agr Food Chem 2005;53 (25):9639-9649.

[49] Kanitskaya LV, Rokhin AV, Kushnarev DF, Kalabin GA. Chemical structure of wheat dioxane lignin : 1H and 13C NMR study. Vysokomol soedin 1998;40 (5):800-805.

[50] Guerra A, Filpponen I, Lucia LA, Argyropoulos DS. Comparative evaluation of three lignin isolation protocols for various wood species. J Agr Food Chem 2006;54 (26):9696-9705.

[51] Choi JW, Faix O, Meier D. Characterization of residual lignins from chemical pulps of spruce (Picea abies L.) and beech (Fagus sylvatica L.) by analytical pyrolysisgas chromatography/mass spectrometry. Holzforschung 2001;55 (2):185-192.

[52] Pinto PC, Evtuguin DV, Neto CP. Effect of structural features of wood biopolymers on hardwood pulping and bleaching performance. Ind Eng Chem Res 2005;44 (26):9777-9784.

[53] Jeffries TW. Biodegradation of lignin-carbohydrate complexes. Biodegradation 1990;1 (2-3):163-176.

[54] Lawoko M, Berggren R, Berthold F, Henriksson G, Gellerstedt G. Changes in the lignin-carbohydrate complex in softwood kraft pulp during kraft and oxygen delignification. Holzforschung 2004;58 (6):603–610.

[55] Lawoko M, Henriksson G, Gellerstedt G. Characterization of lignin-carbohydrate complexes from spruce sulfite pulp. Holzforschung 2006;60 (2):162–165.

[56] Lawoko M, Henriksson G, Gellerstedt G. Structural differences between the lignin-carbohydrate complexes present in wood and in chemical pulps. Biomacromolecules 2005;6 (6):3467-3473.

[57] Willfoer S, Sundberg A, Hemming J, Holmbom B. Polysaccharides in some industrially important softwood species. Wood Sci Technol 2005;39 (4):245-257.

[58] Willfoer S, Sundberg A, Pranovich A, Holmbom B. Polysaccharides in some industrially important hardwood species. Wood Sci Technol 2005;39 (8):601-617.

[59] Jacobs A, Dahlman O. Characterization of the molar masses of hemicelluloses from wood and pulps employing size exclusion chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Biomacromolecules 2001;2 (3):894-905.

[60] Klemm D, Heublein B, Fink H-B, Bohn A. Cellulose: Fascinating biopolymer and sustainable raw material. Angew Chem 2005;44 (22):3358-3393.

[61] Larsson PT, Wickholm K, Iversen T. A CP/MAS ^{13}C NMR investigation of molecular ordering in celluloses. Carbohyd Res 1997;302 (1-2):19-25.

[62] Larsson PT, Hult EL, Wickholm K, Pettersson E, Iversen T. CP/MAS ^{13}C-NMR spectroscopy applied to structure and interaction studies on cellulose I. Solid State Nucl Mag 1999;15 (1):31-40.

[63] Wickholm K, Larsson PT, Iversen T. Assignment of non-crystalline forms in cellulose I by CP/MAS carbon-13 NMR spectroscopy. Carbohyd Res 1998;312 (3):123-129.

[64] Pu Y, Ziemer C, Ragauskas AJ. CP/MAS ^{13}C NMR analysis of cellulase treated bleached softwood kraft pulp. Carbohyd Res 2006;341 (5):591-597.

[65] Sannigrahi P, Ragauskas AJ, Miller SJ. Effects of two-stage dilute acid pretreatment on the structure and composition of lignin and cellulose in loblolly pine. Bioenerg Res 2008;1 (3-4):205-214.

[66] Nishiyama Y, Sugiyama J, Chanzy H, Langan P. Crystal structure and hydrogen bonding system in cellulose I_α from synchrotron x-ray and neutron fiber diffraction. J Am Chem Soc 2003;125 (47):14300-14306.

[67] Browning BL. Methods in wood chemistry. John Wiley: New York, 1967. pp. 387-414.

[68] Brage C, Eriksson T, Gierer J. Reactions of chlorine dioxide with lignins in unbleached pulps: part I. Holzforschung 1991;45 (1):23-30.

[69] Ahlgren PA, Goring DAI. Removal of wood components during chlorite delignification of black spruce. Can J Chem 1971;49 (8):1272-1275.

[70] Kumar R, Mago G, Balan V, Wymand CE. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresource Technol 2009;100 (17):3948-3962.

[71] Hubbell CA, Ragauskas AJ. Effect of acid-chlorite delignification on cellulose degree of polymerization. Bioresource Technol 2010;101 (19):7410-7415.

[72] Pan X, Xie D, Kang KY, Yoon SL, Saddler JN. Effect of organosolv ethanol pretreatment variables on physical characteristics of hybrid poplar substrates. Appl Biochem Biotech 2007;137-140 (1-12):367-377.

[73] Martínez JM, Reguant J, Montero MÁ, Montané D, Salvadó J, Farriol X. Hydrolytic pretreatment of softwood and almond shells. Degree of polymerization and enzymatic digestibility of the cellulose fraction. Ind Eng Chem Res 1997;36 (3):688-696.

[74] Jahan MS, Mun SP. Studies on the macromolecular components of nonwood available in Bangladesh. Ind Crop Prod 2009;30 (3):344-350.

[75] Sweet MS, Winandy JE. Influence of degree of polymerization of cellulose and hemicellulose on strength loss in fire-retardant-treated southern pine. Holzforschung 1999;53 (3):311-317.

[76] Jahan MS, Mun SP. Effect of tree age on the cellulose structure of Nalita wood (*Trema orientalis*). Wood Sci Technol 2005;39 (5):367-373.

[77] Coffey DG, Bell DA, Henderson A. Cellulose and cellulose derivatives. In: Food polysaccharides and their applications. Stephen AM, editors. Marcel Dekker: New York City, New York, 1995. pp. 125.

[78] Sjöholm E. Size exclusion chromatography of cellulose and cellulose derivatives In: Handbook of size exclusion chromatography and related techniques. Wu C, editors. Marcel Dekker: New York, 2004. pp. 311-354.

[79] Zyl JDv. Problem areas in the isolation of cellulose from wood for investigating its molecular properties. Pap Puu 1986;4 320-324.

[80] Klemn D, Philipp B, Heinze T, Heinze U, Wagenknecht W. Analytical methods in cellulose chemistry. In: Fundamentals and analytical methods. Wiley-VCH: New York, 1998. pp. 167-247.

[81] Kleman-Leyer K, Agosin E, Conner AH, Kirk TK. Changes in molecular size distribution of cellulose during attack by white rot and brown rot fungi. Appl Environ Microb 1992;58 (4):1266-1270.

[82] Cohen R, Jensen KA, Houtman CJ, Hammel KE. Significant levels of extracellular reactive oxygen species produced by brown rot basidiomycetes on cellulose. FEBS Lett 2002;531 (3):483-488.

[83] Xu W, Reddy N, Yang Y. Extraction, characterization and potential applications of cellulose in corn kernels and Distillers' dried grains with solubles (DDGS). Carbohyd Polym 2009;76 (4):521-527.

[84] Krässig HA. Cellulose: structure, accessibility, and reactivity. Gordon and Breach Science Publishers: Amsterdam, 1993. pp. 257.

[85] Zhang YP, Lynd LR. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnol Bioeng 2004;88 (7):797-824.

[86] Dupont AL, Mortha G. Comparative evaluation of size-exclusion chromatography and viscometry for the characterisation of cellulose. J Chromatogr A 2004;1026 (1-2):.

[87] Snyder JL, Timell TE. Molecular properties of native balsam fir cellulose. Sven papperstidn 1955;58 851-859.

[88] Timell TE. Chain length and chain-length distribution of native white spruce cellulose. Pulp Paper Mag Can 1955;56 104-114.

[89] Timell TE. Molecular properties of seven native wood celluloses. Tappi J 1957;40 25-29.

[90] Timell TE. Molecular weight and polymolecularity of white birch celluloses. Sven papperstidn 1956;59 1-11.

[91] Puri VP. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. Biotechnol Bioeng 1984;26 (10):1219-1222.

[92] Albersheim P, Darvill A, Roberts K, Sederoff R, Staehelin A. The structural polysaccharides of the cell wall and how they are studied. In: Plant cell walls. Garland Science: New York, 2010. pp. 43-66.

[93] Somerville C. Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol 2006;22 53-78.

[94] Haigler CH. Substrate supply for cellulose synthesis and its stress sensitivity in the cotton fiber. In: Cellulose: molecular and structural biology: selected articles on the synthesis, structure, and applications of cellulose. Brown RM Jr; Saxena IM, editors. Springer: The Netherlands, 2007. pp. 147-163.

[95] Kleman-Leyer KM, Siika-Aho M, Teeri TT, Kirk TK. The cellulases endoglucanase I and cellobiohydrolase II of *Trichoderma reesei* act synergistically to solubilize native cotton cellulose but not to decrease its molecular size. Appl Environ Microb 1996;62 (8):2883-2887.

[96] Henniges U, Kloser E, Patel A, Potthast A, Kosma P, Fischer M, Fischer K, Rosenau T. Studies on DMSO-containing carbanilation mixtures: chemistry, oxidations and cellulose integrity. Cellulose 2007;14 (5):497-511.

[97] Jahana MS, Munb SP. Characteristics of dioxane lignins isolated at different ages of Nalita wood (*Trema orientalis*). J Wood Chem Technol 2007;27 (2):83-98.

[98] Håkansson H, Ahlgren P, Germgård U. The degree of disorder in hardwood kraft pulps studied by means of LODP. Cellulose 2005;12 (3):327-335.

[99] Battista A, Coppicic S, Howsmon JA, Morehead FF, Sisson WA. Level-off degree of polymerization: relation to polyphase structure of cellulose fibers. Ind Eng Chem 1956;48 (2):333-335.

[100] Håkansson H, Ahlgren P. Acid hydrolysis of some industrial pulps: effect of hydrolysis conditions and raw material. Cellulose 2005;12 (2):177-183.

[101] McCormick CL, Callais PA, Hutchinson Jr. BH. Solution studies of cellulose in lithium chloride and *N*,*N*-dimethylacetamide. Macromolecules 1985;18 (12):2394-2401.

[102] Yanagisawa M, Isogai A. SEC-MALS-QELS Study on the molecular conformation of cellulose in LiCl/amide solutions. Biomacromolecules 2005;6 (3):1258-1265.

[103] Röder T, Morgenstern B, Schclosky N, Glatter O. Solutions of *N*,*N*-dimethylacetamide/lithium chloride studied by light scattering methods. Polymer 2001;42 (16):6765-6773.

[104] Sjöholm E, Gustafsson K, Eriksson B, Brown W, Colmsjö A. Aggregation of cellulose in lithium chloride/*N*,*N*-dimethylacetamide. Carbohyd Polym 2000;41 (2):153-161.

[105] Tamai N, Tatsumi D, Matsumoto T. Rheological properties and molecular structure of tunicate cellulose in LiCl/1,3-Dimethyl-2-imidazolidinone. Biomacromolecules 2004;5 (2):422-432.

[106] Sjöholm E, Gustafsson K, Pettersson B, Colmsjö A. Characterization of the cellulosic residues from lithium chloride/*N*,*N*-dimethylacetamide dissolution of softwood kraft pulp. Carbohyd Polym 1997;32 (1):57-63.

[107] Potthast A, Rosenau T, Sixtab H, Kosmaa P. Degradation of cellulosic materials by heating in DMAc/LiCl. Tetrahedron Lett 2002;43 (43):7757-7759.

[108] Yanagisawa M, Isogai A. Size exclusion chromatographic and UV-VIS absorption analyses of unbleached and bleached softwood kraft pulps using LiCl/1,3-dimethyl-2-imidazolidinone as a solvent. Holzforschung 2007;61 (3):236-241.

[109] Yanagisawa M, Shibata I, Isogai A. SEC-MALLS analysis of softwood kraft pulp using LiCl/1,3-dimethyl-2-imidazolidinone as an eluent. Cellulose 2005;12 (2):151-158.

[110] Isogai T, Yanagisawa M, Isogai A. Degrees of polymerization (DP) and DP distribution of dilute acid-hydrolyzed products of alkali-treated native and regenerated celluloses. Cellulose 2008;15 (6):815-823.

[111] Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technol 2005;96 (6):673-686.

[112] Chang VS, Burr B, Holtzapple MT. Lime pretreatment of switchgrass. Appl Biochem Biotech 1997;63-65 (1):3-19.

[113] Chang VS, Nagwani M, Holtzapple MT. Lime pretreatment of crop residues bagasse and wheat straw. Appl Biochem Biotech 1998;74 (3):135-159.

[114] Varga E, Szengyel Z, Réczey K. Chemical pretreatment of corn stover for enhancing enzymatic digestibility. Appl Biochem Biotech 2002;98-100 (1-9):73-87.

[115] Chang VS, Nagwani M, Kim CH, Holtzapple MT. Oxidative lime pretreatment of high-lignin biomass. Appl Biochem Biotech 2001;94 (1):1-28.

[116] Kaar WE, Holtzapple MT. Using lime pretreatment to facilitate the enzymatic hydrolysis of corn stover. Biomass Bioenerg 2000;18 (3):189-199.

[117] Wyman C, Dale BE, Elander RT, Holtzapple MT, Landisch M, Lee YY. Coordinated development of leading biomass pretreatment technologies. Bioresource Technol 2005;96 (18):1959-1966.

[118] Chang VS, Karr WE, Burr B, Holtzapple MT. Simultaneous saccharification and fermentation of lime-treated biomass. Biotechnol Lett 2001;23 (16):1327-1333.

[119] Zheng Y, Pan Z, Zhang R. Overview of biomass pretreatment for cellulosic ethanol production. Int J Agric Biol Eng 2009;2 (3):51－68.

[120] Nguyen QA, Tucker MP, Boynton BL, Keller FA, Schell DJ. Dilute acid pretreatment of softwoods. Appl Biochem Biotech 1998;70-72 (1):77-87.

[121] Schell DJ, Farmer J, Newman M, MCMILLAN JD. Dilute–sulfuric acid pretreatment of corn stover in pilot-scale reactor. Appl Biochem Biotech 2003;105 (1-3):69-85.

[122] Lloyd TA, Wyman CE. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresource Technol 2005;96 (18):1967-1977.

[123] Yang B, Wyman CE. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnol Bioeng 2004;86 (1):88-95.

[124] Hinman ND, Schell DJ, Riley CJ, Bergeron PW, Walter PJ. Preliminary estimate of the cost of ethanol production for SSF technology. Appl Biochem Biotechnol 1992;34-35 (1):639–649.

[125] Lu XB, Zhang YM, Yang J, Liang Y. Enzymatic hydrolysis of corn stover after pretreatment with dilute sulfuric acid. Chem Eng Technol 2007;30 (7):938–944.

[126] Chung YC, Bakalinsky A, Penner MH. Enzymatic saccharification and fermentation of xylose-optimized dilute acid–treated lignocellulosics. Appl Biochem Biotech 2005;121-124 (1-3):947-961.

[127] Yang B, Wyman CE. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng 2006;94 (4):611-617.

[128] Selig MJ, Viamajala S, Decker SR, Tucker MP, Himmel ME, Vinzant TB. Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose. Biotechnol Progr 2007;23 (6):1333-1339.

[129] Pan X, Gilkes N, Kadla K, Pye K, Saka S, Gregg D, Ehara K, Xie D, Lam D, Saddler J. Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: Optimization of process yields. Biotechnol Bioeng 2006;94 (5):851-861.

[130] Pan X, Arato C, Gilkes N, Gregg D, Mabee W, Pye K, Xiao Z, Zhang X, Saddler J. Biorefining of softwoods using ethanol organosolv pulping: Preliminary evaluation of process streams for manufacture of fuel-grade ethanol and co-products. Biotechnol Bioeng 2005;90 (4):473-481.

[131] Zhao X, Cheng K, Liu D. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl Microbiol Biot 2009;82 (5):815-827.

[132] Pan X, Xie D, Yu RW, Lam D, Saddler J. Pretreatment of lodgepole pine killed by mountain pine beetle using the ethanol organosolv process: Fractionation and process optimization. Ind Eng Chem Res 2007;46 (8):2609-2617.

[133] Brosse N, Sannigrahi P, Ragauskas A. Pretreatment of *Miscanthus x giganteus* using the ethanol organosolv process for ethanol production. Ind Eng Chem Res 2009;48 (18):8328–8334.

[134] Pan X, Xie D, Yu RW, Saddler JN. The bioconversion of mountain pine beetle-killed lodgepole pine to fuel ethanol using the organosolv process. Biotechnol Bioeng 2008;101 (1):39-48.

[135] Pan X, Kadla JF, Ehara K, Gilkes N, Saddler JN. Organosolv ethanol lignin from hybrid poplar as a radical scavenger: Relationship between lignin structure, extraction conditions, and antioxidant activity. J Agr Food Chem 2006;54 (16):5806-5813.

[136] Palmqvist E, Han-Hägerdal B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresource Technol 2000;74 (1):25-33.

[137] Chheda JN, Román-Leshkov Y, Dumesic JA. Production of 5-hydroxymethylfurfural and furfural by dehydration of biomass-derived mono- and poly-saccharides. Green Chem 2007;9 (4):342-350.

[138] Sun Y, Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technol 2002;83 (1):1-11.

[139] Palmqvist E, Han-Hägerdal B. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresource Technol 2000;74 (1):17-24.

[140] Jönsson LJ, Palmqvist E, Nilvebrant NO, Han-Hägerdal B. Detoxification of wood hydrolysates with laccase and peroxidase from white-rot fungus *Trametes versicolor*. Appl Microbiol Biot 1998;49 (6):691-697.

[141] Sun XF, Sun RC, Fowler P, Baird MS. Isolation and characterization of cellulose obtained by a two-stage treatment with organosolv and cyanamide activated hydrogen peroxide from wheat straw. Carbohyd Polym 2004;55 (4):379-391.

[142] Väljamäe P, Pettersson G, Johansson G. Mechanism of substrate inhibition in cellulose synergistic degradation. Eur J Biochem 2001;268 (16):4520-4526.

[143] Jeoh T, Ishizawa CI, Davis MF, Himmel ME, Adney WS, Johnson DK. Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. Biotechnol Bioeng 2007;98 (1):112-122.

[144] Chang VS, Holtzapple MT. Fundamental factors affecting biomass enzymatic reactivity. Appl Biochem Biotech 2000;84-86 (1-9):5-37.

[145] Zhu L, O'Dwyer JP, Chang VS, Granda CB, Holtzapple MT. Structural features affecting biomass enzymatic digestibility. Bioresource Technol 2008;99 (9):3817-3828.

[146] Tanaka M, Ikesaka M, Matsuno R, Converse AO. Effect of pore size in substrate and diffusion of enzyme on hydrolysis of cellulosic materials with cellulases. Biotechnol Bioeng 1988;32 (5):698-706.

[147] Ishizawa CI, Davis MF, Schell DF, Johnson DK. Porosity and its effect on the digestibility of dilute sulfuric acid pretreated corn stover. J Agr Food Chem 2007;55 (7):2575-2581.

[148] Hendriks ATWM, Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technol 2009;100 (1):10-18.

[149] Grethlein HE. The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. Nat Biotechnol 1985;3 155-160.

[150] Thompson DN, Chen HC, Grethlein HE. Comparison of pretreatment methods on the basis of available surface area. Bioresource Technol 1992;39 (2):155-163.

[151] Grous WR, Converse AO, Grethlein HE. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. Enzyme Microb Tech 1986;8 (5):274-280.

[152] Gharpuwy MM, Lee Y-H, Fan LT. Structural modification of lignocellulosics by pretreatments to enhance enzymatic hydrolysis. Biotechnol Bioeng 1983;25 (1):157–172.

[153] TAPPI Test Methods. Solvent extractives of wood and pulp. 2007.

[154] Vuorinen T, Alén R. Carbohydrates. In: Analytical Methods in Wood Chemistry, Pulping, and Papermaking. Sjöström E, Alén R, editors. Springer-Verlag: Berlin, Germany, 1998. pp. 38-40.

[155] Zhang D, Pu Y, Chai XS, Naithani V, Jameel H, Ragauskas AJ. Elucidating carboxylic acid profiles for extended oxygen delignification of high-kappa softwood kraft pulps. Holzforschung 2006;60 (2):123-129.

[156] Liitiä T, Maunu SL, Hortling B, Tamminen T, Pekkala O, Varhimo A. Cellulose crystallinity and ordering of hemicelluloses in pine and birch pulps as revealed by solid-state NMR spectroscopic methods. Cellulose (Dordrecht, Netherlands) 2003;10 (4):307-316.

[157] Guerra A, Mendonca R, Ferraz A, Lu F, Ralph J. Structural characterization of lignin during *Pinus taeda* wood treatment with *Ceriporiopsis subvermispora*. Appl Environ Microb 2004;70 (7):4073-4078.

[158] Holtman KM, Chang H, Jameel H, Kadla J. Quantitative ^{13}C NMR characterization of milled wood lignins isolated by different milling techniques. J Wood Chem Technol 2006;26 (1):21-34.

[159] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. Determination of structural carbohydrates and lignin in biomass. Laboratory Analytical Procedures; National Renewable Energy Laboratory: Golden, CO. 2008.

[160] Allison L, Ragauskas AJ, Hsieh JS. Metal profiling of southeastern US softwood (SW) and hardwood (HW) furnish. Tappi J. 2000;83 (8):97.

[161] Adney B, Baker J. Measurement of cellulase activities. Laboratory Analytical Procedures; National Renewable Energy Laboratory: Golden, CO. 1996.

[162] Granata A, Argyropoulos DS. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. J Agr Food Chem 1995;43 (6):1538-1544.

[163] Pu Y, Ragauskas AJ. Structural analysis of acetylated hardwood lignins and their photoyellowing properties. Can J Chem 2005;83 (12):2132-2139.

[164] Pu Y, Chen F, Ziebell A, Davison BH, Ragauskas AJ. NMR characterization of C3H and HCT down-regulated alfalfa lignin. Bioenerg Res 2009;2 (4):198-208.

[165] Lundquist K. Proton (^{1}H) NMR spectroscopy. In: Methods in Lignin Chemistry. Lin SY, Dence CW, editors. Springer-Verlag: Berlin, Germany, 1992. pp. 242-249.

[166] Nagy M, David K, Britovsek GJP, Ragauskas AJ. Catalytic hydrogenolysis of ethanol organosolv lignin. Holzforschung 2009;63 (5):513-520.

[167] Schubert C. Can biofuels finally take center stage? Nat Biotechnol 2006;24 (7):777-784.

[168] Berg A, Navarrete P, Olave L. Biochemicals and Standardized Solid Fuels from Radiata Pine Bark. In: 15th European Biomass Conference & Exhibition, Berlin, Germany, 2007.

[169] TAPPI Test Methods. Gross heating value of black liquor. 2006.

[170] Scott RW. Colorimetric determination of hexuronic acids in plant materials. Anal Chem 1979;51 (7):936-941.

[171] White RH. Effect of lignin content and extractives on the higher heating value of wood. Wood Fiber Sci 1986;19 (4):446-452.

[172] Guillermo Avila J, de Liverant JG, Martinez Andres, Martinez G, Munoz JL, Arciniegas Amira, Romo de Vivar A. Mode of action of *Buddleja cordata* verbascoside against *Staphylococcus aureus*. J Ethnopharmacol 1999;66 (1):75-78.

[173] Lennholm H, Larsson T, Iversen T. Determination of cellulose I_α and I_β in lignocellulosic materials. Carbohyd Res 1994;261 (1):119-131.

[174] Holtman KM, Chang H, Kadla JF. Solution-state nuclear magnetic resonance study of the similarities between milled wood lignin and cellulolytic enzyme lignin. J Agr Food Chem 2004;52 (4):720-726.

[175] Crestini C, Argyropoulos DS. Structural analysis of wheat straw lignin by quantitative ^{31}P and 2D NMR spectroscopy. The occurrence of ester bonds and α-O-4 substructures. J Agr Food Chem 1997;45 (4):1212-1219.

[176] El Hage R, Brosse N, Chrusciel L, Sanchez C, Sannigrahi P, Ragauskas A. Characterization of milled wood lignin and ethanol organosolv lignin from *miscanthus*. Polym Degrad Stabil 2009;94 (10):1632-1638.

[177] Akim LG, Argyropoulos DS, Jouanin L, Leplé J, Gilles Pilate G, Brigitte Pollet B, Lapierre C. Quantitative ^{31}P NMR Spectroscopy of lignins from transgenic poplars. Holzforschung 2001;55 (4):386-390.

[178] Schülein M. Protein engineering of cellulases. Biochim Biophys Acta 2000;1543 (2):239-252.

[179] Araque E, Parra C, Freer J, Contreras D, Rodríguez J, Mendonça R, Baeza J. Evaluation of organosolv pretreatment for the conversion of *Pinus radiata* D. Don to ethanol. Enzyme Microb Tech 2008;43 (2):214-219.

[180] Esteghlalian A, Hashimoto AG, Fenske JJ, Penner MH. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar, and switchgrass. Bioresource Technol 1997;59 (2-3):129-136.

[181] Söderström J, Pilcher L, Galbe M, Zacchi G. Two-step steam pretreatment of softwood by dilute H_2SO_4 impregnation for ethanol production. Biomass Bioenerg 2003;24 (6):475-486.

[182] Ewanick S, Bura R, Saddler JN. Acid-catalyzed steam pretreatment of lodgepole pine and subsequent enzymatic hydrolysis and fermentation to ethanol. Biotechnol Bioeng 2007;98 (4):737-746.

[183] Yang ST. Bioprocessing for value-added products from renewable resources: new technologies and applications. Elsevier: New York, USA, 2007. pp. 1-24.

[184] Dai Z, Hooker BS, Quesenberry RD, Gao J. Expression of *Trichoderma reesei* exo-cellobiohydrolase I in transgenic tobacco leaves and calli. Appl Biochem Biotech 1999;79 (13):689-699.

[185] Horn SJ, Sikorski P, Cederkvist JB, Vaaje-Kolstad G, Sørlie M, Synstad B, Vriend G, Vårum KM, Eijsink VGH. Costs and benefits of processivity in enzymatic degradation of recalcitrant polysaccharides. P Natl Acad Sci USA 2006;103 (48):18089-18094.

[186] Yoshida M, Liu Y, Uchida S, Kawarada K, Ukagami Y, Ichinose H, Kaneko S, Fukuda K. Effects of cellulose crystallinity, hemicellulose, and lignin on the enzymatic hydrolysis of *miscanthus sinensis* to monosaccharides. Biosci Biotech Bioch 2008;72 (3):805–810.

[187] Hallac BB, Sannigrahi P, Pu Y, Ray M, Murphy RJ, Ragauskas AJ. Biomass characterization of *Buddleja davidii*: A potential feedstock for biofuel production. J Agr Food Chem 2009;57 (4):1275-1281.

[188] Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D. Determination of sugars, byproducts, and degradation products in liquid fraction process samples.

Laboratory Analytical Procedures; National Renewable Energy Laboratory: Golden, CO. 2006.

[189] Martinez A, Rodriguez ME, York SW, Preston JF, Ingram LO. Use of UV absorbance to monitor furans in dilute acid hydrolysates of biomass. Biotechnol Progr 2000;16 (4):637-641.

[190] Öhgren K, Bura R, Saddler J, Zacchi G. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresource Technol 2007;98 (13):2503-2510.

[191] Baeza J, Fernández AM, Freer J, Pedreros A, Schmidt E, Durán N. Organosolv-pulping III: the influence of formic acid delignification on the enzymic hydrolysis of *Pinus radiata* D. Don sawdust. Appl Biochem Biotech 1991;31 (3):273-282.

[192] Bergenstråhle M, Wohlert J, Larsson PT, Mazeau K, Berglund LA. Dynamics of cellulose-water interfaces: NMR spin-lattice relaxation times calculated from atomistic computer simulations. J Phys Chem B 2008;112 (9):2590-2595.

[193] Gellerstedt G, Li J, Eide I, Kleinert M, Barth T. Chemical structures present in biofuel obtained from lignin. Energ Fuel 2008;22 (6):4240–4244.

[194] Hallac BB, Sannigrahi P, Pu Y, Ray M, Murphy RJ, Ragauskas AJ. Effect of ethanol organosolv pretreatment on enzymatic hydrolysis of *Buddleja davidii* stem biomass. Ind Eng Chem Res 2010;49 (4):1467-1472.

[195] Xia Z, Akim LG, Argyropoulos DS. Quantitative ^{13}C NMR analysis of lignins with internal standards. J Agr Food Chem 2001;49 (8):3573-3578.

[196] Pu Y, Anderson S, Lucia L, Ragauskas AJ. Investigation of the photooxidative chemistry of acetylated softwood lignin. J Photoch Photobio A 2004;163 (1-2):215-221.

[197] Sannigrahi P, Ragauskas AJ, Miller SJ. Lignin structural modifications resulting from ethanol organosolv treatment of loblolly pine. Energ Fuel 2010;24 (1):683-689.

[198] Baptista C, Robert D, Duarte AP. Relationship between lignin structure and delignification degree in *Pinus pinaster* kraft pulps. Bioresource Technol 2008;99 (7):2349–2356.

[199] Jiang Z, Argyropoulos DS. Isolation and characterization of residual lignins in kraft pulps. J Pulp Pap Sci 1999;25 (1):25-29.

[200] Lindner A, Wegener G. Characterization of lignins from organosolv pulping according to the organocell process. Part 1: Elemnetal analysis, nonlignin portions and fuctional groups. J Wood Chem Technol 1988;8 (3):323-340.

[201] Li J, Henriksson G, Gellerstedt G. Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. Bioresource Technol 2007;98 (16):3061-3068.

[202] McDonough TJ. The chemistry of organosolv delignification Tappi J 1993;76 (8):186-193.

[203] Villaverde JJ, Li J, Ek M, Ligero P, De Vega A. Native lignin structure of *miscanthus x giganteus* and its changes during acetic and formic acid fractionation. J Agr Food Chem 2009;57 (14):6262-6270.

[204] Robert D. Carbon-13 nuclear magnetic resonance spectroscopy. In: Methods in lignin chemistry. Lin SY; Dence CW, editors. Springer-Verlag: New York, 1992. pp. 578.

[205] del Río JC, Rencoret J, Marques G, Gutiérrez A, Ibarra D, Santos JI, Jiménez-Barbero J, Zhang L, Martínez ÁT. Highly acylated (acetylated and/or *p*-coumaroylated) native lignins from diverse herbaceous plants. J Agr Food Chem 2008;56 (20):9525-9534.

[206] Kringstad KP, Mörck R. ^{13}C-NMR spectra of kraft lignins. Holzforschung 1983;37 (5):237-244.

[207] Kishimoto T, Ueki A, Takamori H, Uraki Y, Ubukata M. Delignification mechanism during high-boiling solvent pulping. Part 6: Changes in lignin structure analyzed by ^{1}H-^{13}C correlation 2-D NMR spectroscopy Holzforschung 2004;58 (4):355-362.

[208] Li S, Lundquist K, Westermark U. Cleavage of arylglycerol β-aryl ethers under neutral and acid conditions. Nord Pulp Pap Res J 2000;15 (4):292-299.

[209] Capanema EW, Balakshin MY, Chen C, Gratzl JS, Gracz H. Structural analysis of residual and technical lignins by ^{1}H-^{13}C correlation 2D NMR-spectroscopy. . Holzforschung 2001;55 (3):302-308.

[210] Bardet M, Robert DR, Lundquist K. On the reactions and degradation of the lignin during steam hydrolysis of aspen wood. sven papperstidn 1985;88 (6):R61-R67.

[211] Li S, Lundquist K. Acid reactions of lignin models of β-5 type. Holzforschung 1999;53 (1):39-42.

[212] Ray M, Kleist G, Murphy R. Decay assessment in a hardwood handrail at the South Bank, London. J I Wood Sci 2005;17 (1):51-58.

[213] Srebotnik E, Messner K. A simple method that uses differential staining and light microscopy to assess the selectivity of wood delignification by white rot fungi. Appl Environ Microb 1994;60 (4):1383-1386.

[214] Hernández M, Hernández-Coronado MJ, Pérez MI, Revilla E, Villar JC, Ball AS, Viikari L, Arias ME. Biomechanical pulping of spruce wood chips with *Streptomyces cyaneus* CECT 3335 and handsheet characterization. Holzforschung 2005;59 (2):173-177.

[215] Donohoe BS, Decker SR, Tucker MP, Himmel ME, Vinzant TB. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. Biotechnol Bioeng 2008;101 (5):913-925.

[216] Ishizawa CI, Jeoh T, Adney WS, Himmel ME, Johnson DK, Davis MF. Can delignification decrease cellulose digestibility in acid pretreated corn stover? Cellulose 2009;16 (4):677-686.

[217] Martínez ÁT, Speranza M, Ruiz-Dueñas FJ, Ferreira P, Camarero S, Guillén F, Martínez MJ, Gutiérrez A, del Río JC. Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. Int Microbiol 2005;8 (3):195-204.